

ETHYLENE BIOSYNTHESIS, PERCEPTION, AND SIGNALING-RELATED GENE  
EXPRESSION DURING PAPAYA FRUIT (*Carica papaya* L.) RIPENING

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## Abstract

A gaseous plant growth regulator, ethylene, plays an important role during plant growth and development and includes ethylene dependent fruit ripening. Fruit ripening is a series of biochemical, physiological and structural events that lead to maturity. Papaya (*Carica papaya*) is a typical climacteric fruit that which performs dramatic changes in color, texture, and flavor during fruit ripening. Fruit ripening process was considered highly related to the biosynthesis of ethylene which is mainly controlled by the SAM (S-Adenosyl methionine synthetase, Methionine adenosyltransferase), ACS (1-aminocyclopropane-1-carboxylic acid synthase), and ACO (1-aminocyclopropane-1-carboxylic acid oxidase) genes. In addition, the ethylene receptors in *Arabidopsis* and tomato have been shown to be involved in effecting fruit development and the timing of fruit ripening, respectively. Ethylene signaling transduction gene expressions, such as CTR (Constituted Triple Response), EIN2 (Ethylene Insensitivity 2), EIN3/EILs (Ethylene Insensitivity 3 and Ethylene Insensitivity 3- Like proteins) and ERF (Ethylene Response Factors) are also involved in the ripening processing.

The full sequencing of the papaya genome, coupled with microarray technology, provides a chance to determine the expressions of genes at specific fruit developmental stages. Thirty-four genes involved in ethylene biosynthesis (SAM, ACS, ACO, and ETO), perception (ETR) and the signaling transduction pathway (RAN, CTR, EIN2, EIN3/EIL1, and ERF) were selected from 24,421 predicted genes in papaya genome. Four developmental stages: mature green, the 25% color, 80% color and 100 color stage were investigated. The ethylene biosynthesis genes seemed to be expressed before the

initiation of fruit ripening, and declined once the System 2 ethylene production was started. The ethylene receptors have been shown to be involved in the regulation of tomato fruit ripening. Our results showed fewer number of ethylene receptors than in tomato and *Arabidopsis* and also showed a possible role in controlling the initiation of papaya fruit ripening. More genes related to the ethylene signaling transduction pathway did not change in expression level during fruit maturation and ripening, except for CTR and RAN. CTR1 and RAN are considered as negative regulator in the signaling pathway and a copper transporter associated with ethylene receptor respectively.

In papaya, changes in sugar content, production of flavor constituents and rapid pulp softening during ripening can affect the quality and cause serious postharvest losses during transportation and storage. Knowing the ethylene-related gene expression during papaya fruit development and ripening may be helpful in regulating the timing of ripening, in order to control the fruit quality and reduce the postharvest losses.

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# **1. Introduction**

## **1.1 Ethylene and Fruit Ripening**

Ethylene is a simple gaseous molecule, produced during plant development and in response to biotic and abiotic stimuli. Almost all higher plant organs can synthesize ethylene, though the production rate depends on the type of tissue and the stage of the development (Abeles et al., 1992). Ethylene freely diffuses through tissues and affects other tissues or organs at low active concentrations. In plants, ethylene has been recognized as an important plant growth regulator (PGR) that operates throughout the entire plant's life. This PGR plays an important roles in germination, cell expansion, cell differentiation, flowering, senescence, abscission, and fruit ripening (Abeles et al., 1992; Bleecker and Kende, 2000; Lin, et al., 2009).

Fleshy fruit ripening is a genetically programmed event, and corresponds to a series of biochemical, physiological and structural changes that cause fruit to reach its mature. Although the processes may vary among species, most fruit display changes in flavor, texture, color, and aroma. Fruits can be divided into two groups based on the amount and pattern of ethylene produced during ripening (Inaba, 2007). Climacteric fruits are described as having a respiration peak that appears before ethylene production and ripening initiation, whereas non-climacteric fruits do not exhibit a burst of ethylene biosynthesis at the onset of ripening (Giovannoni, 2001; Lelièvre, et al., 1997; White, 2002; Yokotani, 2009).

## 1.2 Ethylene Biosynthesis Pathway

It has been proposed that two systems for ethylene synthesis regulation occur in higher plants based on the level of ethylene production during fruit ripening (Chang, et al., 2008; McMurchie, et al., 1972; Nakatsuka, 1998; Schaller and Kieber, 2002). Ethylene production system 1 maintains the basal level of ethylene production in all tissues during vegetative growth, including both climacteric and non-climacteric fruit before the onset of ripening (Barry, et al., 2000; Lelièvre, et al., 1997). In contrast, system 2 represents a massive ethylene production during floral senescence and ripening of climacteric fruit, and ethylene produced by mature climacteric fruit results in autocatalytic ethylene biosynthesis (System 2) and leads to fruit ripening.

Unlike other plant growth regulators, ethylene biosynthesis can be regulated by ethylene itself by both positive and negative feedback. Fruit may show different responses to exogenous ethylene that differ among varieties and developmental stages. Exposing non-climacteric immature fruit to ethylene causes no effect on the amount of ethylene produced, but can accelerate the initiation of fruit ripening (Lelièvre, et al., 1997). However, in immature climacteric fruit, ethylene synthesis can be auto-inhibited by exogenous ethylene (Kevany, et al., 2007; Lelievre, et al., 1997).

The ethylene biosynthesis pathway in higher plants is derived from the amino acid methionine through S-adenosylmethionine (AdoMet), that is converted to 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC synthase (ACS). The last step of ethylene biosynthesis is the formation of ethylene from ACC by ACC oxidase (ACO) (Abeles et al., 1992; Argueso, et al., 2007; Lelièvre, et al., 1997; Yang and Hoffman, 1984).

Ado-Met (S-adenosylmethionine) is converted from the essential amino acid methionine by SAM synthetase (Ado-Met synthetase, S-Adenosyl methionine synthetase, Methionine adenosyltransferase). Ado-Met is not just the precursor for ethylene biosynthesis, it is also the precursor in the biosynthesis of polyamines and biotin in plants (Ravanel et al., 1998; Roeder, et al., 2009; Wang, et al, 2002). In addition, Ado-Met acts as a methyl donor

The conversion of AdoMet to ACC is generally considered the rate-limiting step. However, more evidence suggested that this may not be the rate limiting step in all fruits and that ACO plays an important role in ethylene biosynthesis in tomato and melon fruit, especially during the high ethylene production period (Chang, et al., 2008; Nakatsuk, et al., 1997; Schaller and Kieber, 2002).

Studying the accumulation of ACC during climacteric fruit ripening indicated the relative activities of ACS and ACO, and their suppression by transgenic technology reduces ethylene production (Abeles, et al., 1992; Akisugu, 2007; Lelièvre, et al., 1997). The gene expressions and activities of ACS and ACO both increase during fruit development and maturation, and might indirectly affect fruit ripening by regulating ethylene biosynthesis at certain stages during fruit maturation.

Both ACS and ACO are encoded by multigene families, and the expression is regulated by various different developmental and environmental signals (Chang, 2008; Li, 2010; Schaller and Kieber, 2002). ACS and ACO isogenes may also be responsible for the regulation of ethylene production at different stages of climacteric fruit ripening (Barry, et al., 2000; Chang, et al., 2008; Inaba, 2008; Tatsuki, 2010).

In *Arabidopsis*, eight encoded ACS genes and one additional inactivated *ACS1*

enzyme have been found (Argueso, et al., 2007). At least eight ACS isogenes along with four ACO isogenes have been identified in tomato (*Lycopersicon esculentum*) (Alexander and Grierson, 2002; Barry, et al., 2000; Chang, 2008; Inaba, 2007). Only two tomato ACS isogenes, LeACS2 and LeACS4, and two ACO isogenes, LeACO1 and LeACO4, have been demonstrated to induce the system 2 ethylene production in a positive feedback manner during tomato fruit ripening (Bapat, et al., 2010; Barry, et al., 2000; Inaba, 2007; Tatsuki, 2010; Yokotani, et al., 2009). System 1 ethylene production in tomato is mediated by LeACS1a, LeACS3 positively, and LeACS6, LeACO1 and LeACO4 negatively (Inaba, 2007).

In apple (*Malus domestica*), five ACS isogenes have been identified (Tatsuki, 2010). The expression of MdACS isogenes varies in different cultivars and is affected by environmental factors. MdACS1 is the predominant enzyme in climacteric fruit for System 2 ethylene production, and MdACS3 is expressed for system 1 ethylene production (Sunako, et al., 1999; Tatsuki, 2010). Three ACS isogenes have been identified in peach, but only one ACS isogene, PpACS1, shows an increase in expression during melting peach ripening. However, the expression of PpACS1 does not change and the ethylene production is inhibited during ripening.

### **1.3 Ethylene Reception**

Ethylene functions as a plant growth regulator by being perceived by a family of endoplasmic reticulum-associated membrane proteins: ethylene receptors. These receptors are similar in structure to the bacterial two-component receptors and are mediated by a copper co-factor (Chen et al., 2002; Bleecker and Kende, 2000; Gamble et

al., 1998; Schaller et al., 1995). The ethylene receptors can be divided into two subfamilies: Subfamily I and II, based on the differences in the histidine kinase activities of the receptor protein. The protein histidine kinase activities have been showed to be involved in the signal transduction and this transduction is also similar to that in bacterial two-component system. As with the two-component receptors, the ethylene receptors have three major functional domains: an N-terminal sensor domain for ethylene binding, a histidine kinase domain, and a receiver domain in the C-terminal region (Cancel and Larsen, 2002; Hall and Bleeker, 2003; Huang, et al., 2003; Lelièvre, et al., 1997). The histidine kinase domain in subfamily II lacks one or more amino acids thought to be important for catalytic activity (Tatsuki, 2010). The ethylene receptor family includes ETR1, ERS1, ETR2, ERS2, and EIN4 in *Arabidopsis* (Hua, 1998); and LeETR1, LeETR2, LeETR4, LeETR5, LeETR6, and NR in tomato (Lashbrook, et al., 1998; Tieman and Klee, 1999). In *Arabidopsis*, the functions of subfamily I receptors, ETR1 and ERS1, cannot be replaced by the subfamily II receptors, ETR2, ERS2, and EIN4. However, in tomato, the subfamily II seems more important in fruit ripening with LeETR4 being directly involved. The tomato with antisense LeETR4 leads to earlier fruit ripening that can be restored by the subfamily I ethylene receptor, NR (Kevany, et al., 2007; Tieman, et al., 2000). The single ethylene receptor mutant of *Arabidopsis* does not show obvious effect on ethylene response, which indicates the effects of ethylene receptor are redundant. In tomato, reducing the expression of NR, LeETR1, LeETR2, or LeETR5 does not lead to ethylene related responses during growth and development (Kevany, et al., 2007; Tieman, et al., 2000).

Previous studies in *Arabidopsis* and tomato fruit proved that ethylene receptors are

negative regulators of the ethylene response pathway (Bleecker and Kende, 2000; Hua and Meyerowitz, 1998; Tieman et al., 2000). When ethylene is absent, the ethylene response pathway is suppressed by the activated ethylene receptors. The ethylene response pathway is activated by the binding of ethylene or ethylene-like chemicals to the receptors. Ethylene-like chemicals have similar structures to ethylene and thus can be recognized by the ethylene receptors (Frye, et al., 2001; Tieman et al., 2000). For example, 1-MCP is a compound that inhibits ethylene binding by the ethylene receptors, and is therefore used as ethylene binding inhibitor (Sisler and Serek, 1997).

Earlier experiments demonstrated that ethylene sensitivity is increased by reducing the amount of strawberry (*Fragaria × ananassa*) ethylene receptors (Hua and Meyerowitz, 1998; Tieman et al., 2000). In the non-climacteric fruit, three ethylene receptors have been identified, FaETR1, FaERS1, and FaETR2 (Trainotti et al., 2005). The overall ethylene receptor expression increases during strawberry fruit development (Trainotti et al., 2005). The strawberry subfamily I receptors, FaETR1 and FaERS1, are expressed during early developmental stages and continue to increase during fruit ripening; but FaETR2 which belongs to subfamily II receptor, reaches its highest expression as fruit ripening starts. This result is similar to the functional analysis of ethylene receptor in tomato, where LeETR4 plays a major role in tomato fruit (Tieman et al., 2000).

In climacteric fruit, the ethylene receptors are found to be involved in early fruit development and fruit ripening in muskmelon (Sato-Nara et al., 1999). Furthermore, Kevany et al. (2007) indicated the importance of ethylene receptors in controlling the timing of tomato fruit ripening by looking at ethylene receptors gene expression. The

negative regulation theory, suggests that the ethylene response is reduced by increasing the amount of receptors.

#### **1.4 Ethylene Signal Transduction Pathway**

After ethylene binds to the receptors on the endoplasmic reticulum (ER), the signal is transmitted and activates the downstream ethylene response pathway. The ethylene pathway includes the receptors along with the Raf, a similar protein kinase, and the constitutive triple response (CTR1) gene. Loss-function of CTR1 reduces ethylene sensitivity and results in the constitutive ethylene responses, indicating that CTR1 acts as a negative regulator of the ethylene signal transduction pathway (Adams-Philips, et al., 2004, Huang, et al., 2003). However, the loss of CTR1 function may not cause effects on ethylene response. The signal output from CTR may also be dependent on the interaction with the ethylene receptor (Hall, et al., 2007). In *Arabidopsis*, a single CTR1 has been identified, and it is preferentially associated with subfamily I receptor, ETR1 and ERS1. Loss functions of subfamily I receptors leads to the constitutive ethylene response but only mild responses have been showed in loss-function of subfamily II receptor mutant (Hua and Meyerowitz, 1998; Hall, et al., 2007).

A single CTR1 gene, MdCTR1 has also found in apple (*Malus domestica*) (Cin, et al., 2005; Wiersma, et al., 2007). A decline in MdCTR1 expression may indicate an association between the expression of MdCTR1 and ethylene sensitivity during maturation (Wiersma, et al., 2007). Also, different apple cultivars show different degrees of decrease in MdCTR1 expression during fruit ripening. Based on the cultivar-dependent



variance, suggests multiple CTR1 genes are involved in the ethylene response (Adams-Philips, et al., 2004; Wiersma, et al., 2007).

Unlike *Arabidopsis*, multiple CTR1 genes have been identified in tomato and kiwi (*Actinidia deliciosa*). At least three CTR1 isogenes are found in tomato (Adams-Philips, et al., 2004; Barry and Giovannoni, 2007). The LeCTR1, LeCTR3, and LeCTR5 are identified as being closely related to *Arabidopsis* CTR1, and the functions of these three homologs are complementary. LeCTR1 shows unregulation during fruit ripening (Giovannoni, 2004), while all three CTR1 homologs in tomato are regulated differently by ethylene during ripening (Adams-Phillips et al., 2004; Cin, et al., 2006). Two CTR1-like genes have been identified in kiwifruit (Yin, et al., 2008). The expression of AdCTR1 in flesh increases during fruit ripening, but shown no response to exogenous ethylene treatment. AdCTR2 shows no change during fruit ripening and no responses to 1-MCP treatment. However, ethylene treatment can enhance the expression of AdCTR1 in the core (Yin, et al., 2008).

### **1.5 Transcriptional Factors (Regulators) – EIN2**

Ethylene Insensitivity 2 (EIN2) is a transcription factor, located downstream of CTR1, and it is the central to the ethylene signal transduction response pathway (Jonson and Ecker, 1998). EIN2 may also act as a cross-talk point for other hormone pathway (Adams-Phillips et al., 2004; Zhu, et al., 2006), such as auxin (Fujita and Syono, 1996), abscisic acid (Beaudoin, et al., 2000; Ghassemian, et al., 2000), cytokinin (Cary, et al., 1995), and jasmonate (Penninckx, et al., 1998). However, the actual function of EIN2 remains unclear.

EIN2 is encoded by single gene in *Arabidopsis* and tomato (Klee, 2004). Loss function of EIN2 in *Arabidopsis* shows a similar phenotype as in *etr1* mutant and results in ethylene insensitivity; this indirectly indicates that the EIN2 plays an important role in ethylene signal transduction pathway (Etheridge, et al., 2005; Johnson and Ecker, 1998; Zhu, et al., 2006). *LeEIN2* expression, however, shows no change and is not ethylene-inducible during fruit development, although reducing *LeEIN2* expression delays fruit ripening (Klee, 2004).

#### **1.6 EIN3/EIL (EIN3-like) Genes and ERF (Ethylene Response Factors)**

Downstream of EIN2 is EIN3 (Ethylene Insensitivity 3). EIN3 includes the family of EILs (EIN3-Like proteins) and the ERFs (Ethylene Responsive Factors). EIN3/EILs act as a positive regulator in ethylene response pathway, and mediate the morphological response. EIN3 is recognized as a transcription factor and the termination of ethylene signal transduction pathway (Adams-Philips, et al., 2004; Barry and Giovannoni, 2007; Johnson and Ecker, 1998; Schaller and Kieber, 2002). Not only does ethylene regulate the expression of EIN3, the level of EIN3 protein can also affect its expression. The degradation of EIN3 protein is controlled by ubiquitin/proteasome pathway. The EIN3 protein is easily degraded when ethylene is absent, but EIN3 protein level accumulate in presence of exogenous ethylene (Guo and Ecker, 2003; Klee, 2004).

The loss-function of EIN3 results in ethylene insensitivity while an over-expressed mutant results a constitutive triple response (Barry and Giovannoni, 2007; Chao, et al., 1997). Three EIN3 isogenes have been found in tomato (Klee, 2004; Tieman,

et al., 2001), and all EIN3 genes functionally restore the *Arabidopsis* ein3 mutant (Kless, 2004).

ERFs are a group of transcription factors that are specific to plants. These transcription factors specifically bind to GCC box (Adams-Philips, et al., 2004; Ohme-Takagi and Shinshi, 1995; Tournier, et al., 2003; Wang, et al., 2007). In *Arabidopsis*, 122 ERF genes are predicted. Only a few ERF proteins have been isolated and most of them are recognized as involving stress and hormone responses (Nakano et al., 2006; Tournier, et al., 2003; Wang, et al., 2007).

Based on the structure and the transcriptional activities, the four ERFs in tomato are classified into different classes. LeERF1 (Class I) and LeERF4 (Class III) are activators, and LeERF3 (Class II) are the repressors of ethylene response. LeERF2 has a unique N-terminal motif and likely play an important role in fruit ripening (Tournier, et al., 2003; Wang, et al., 2007). All LeERFs are up-regulated by wounding or exogenous ethylene.

## **1.7 Ethylene Biosynthesis, Perception, and Response Genes in Papaya**

### **1.7.1 Ethylene biosynthesis genes in papaya**

The papaya genome has been sequenced (Ming, et al., 2008). For ethylene biosynthesis, the papaya genome is predicted to have four S-adenosyl-L-methionine synthases (SAM), seven ACC synthases (ACS), and three ACC oxidases (ACO) (Paull, et al., 2008).

### **1.7.2 Ethylene perception in papaya**

The ethylene receptors in tomato are involved in ethylene sensitivity during fruit development and ripening. In papaya, at least three ethylene receptors have been found (CpETR1 to CpETR3) (Paull, et al., 2008). These ethylene receptors contained the expected protein domains for ethylene binding similar to those in *Arabidopsis* and tomato (Klee and Tieman, 2002; Paull, et al., 2008). CpETR1 has 100% homology to cDNA sequence of *Carica papaya* ethylene receptor (AF311942.1) cloned by Dr. Lazan in Malaysia (Paull, et al., 2008; Lazan, et al., unpublished). CpETR2 and CpETR3 are found to be 86% homologous with *Arabidopsis* ETR1 (At1g66340) and have 67% homology with tomato ethylene receptor, respectively. Based on the protein structure, CpETR1 and CpETR2 are grouped into subfamily I, while CpETR3 belonged to subfamily II. A fourth ethylene receptor in the papaya genome is highly expressed during fruit ripening (data unpublished). However, the sequence in the papaya genome is incomplete.

Fewer ethylene receptors are found in papaya than in *Arabidopsis* (five) and in tomato (six), and suggests that the papaya may show higher sensitivity to ethylene (Paull, et al., 2008).

### **1.7.3 Ethylene signal transduction pathway: CTR1 kinase**

As mentioned above, a single CTR1 has been identified in *Arabidopsis*, while three CTR1 isogenes were found in tomato. In papaya, there was at least one CTR1 has been found with 56% homology with LeCTR1 (Paull, et al., 2008).

#### **1.7.4 Ethylene signal transduction pathway: EIN2, EIN3/EIL, and ERF**

A single EIN2 has also been predicted in papaya genome, as in *Arabidopsis*. However, papaya has fewer EIN3/EIL genes. Only four possible EIN3/EIL genes are predicted, while there were at nine in *Arabidopsis* and five in tomato (Binder, et al., 2007; Paull, et al., 2008; Stepanoya and Alonso, 2005).

Eight CpERFs are predicted, all with high homology to ERF sequences in *Arabidopsis*. An additional twelve ERF-like proteins have been predicted in papaya genome that show low homology with ERF and were with incomplete sequences (Paull, et al., 2008).

#### **1.8 Papaya Fruit Ripening**

Papaya (*Carica papaya* L.) is a member of the family *Caricaceae*. It is an important crop that is planted in the tropic worldwide. The fruit is consumed fresh and is also processed to juice and product. As a climacteric fruit, the respiration rate increases at the onset of ripening, and is followed by increased ethylene production (Mitra, 1997; Paull and Chen, 1983). Besides the increases in respiration rate and ethylene production, the papaya fruit ripening involves changes in total soluble solids, pigments, polysaccharide structure, and the fruit texture (Mitra, 1997; Paull and Chen, 1983).

During fruit ripening, the activity of acid cell wall invertase increases that breaks down sucrose to glucose and fructose prior to their uptake and storage within cell. The total nonvolatile acids (citric and malic acids) decrease and reach the lowest level at ripe stage (Ghanta, 1994; Mitra, 1997). The mesocarp turns yellow or red during fruit

development due to the increases in carotenoids and lycopene (red-fleshed type) (Chan, 1983; Mitra, 1997; Selvaraj, et al., 1982). More than 199 volatile components have been identified in papaya (Idstein, 1985; Mitra, 1997) and are responsible for the sweetish aroma of ripe papaya fruit (MacLeo and Pieris, 1983; Mitra, 1997; Morales and Duque, 1987). The amount of volatile components released is usually low, and varies among cultivars and with developmental stage (Flath, et al., 1990, Mitra, 1997).

Rapid pulp softening during fruit ripening is considered to contribute to the major postharvest losses during transportation and storage (Fabi, et al., 2007; Mitra, 1997; Paull and Chen, 1983; Paull, et al., 1997). Cell wall disassembly during papaya fruit ripening involves differential call wall hydrolase inducing endoxylanase gene expression at different developmental stages and the expression patterns vary among cultivars.

### **1.9 Research Objectives and Hypothesis**

The postharvest losses of papaya fruit ranged from 40-100%. Fruit ripening involves changes in sugar content, production of flavor constituents, and softening of mesocarp and endocarp. All these changes affect postharvest losses due to diseases, physiological disorders, and mechanical damages (Paull, et al., 1997). To understand the ethylene biosynthesis and signaling pathway in papaya fruit may key in the control of the timing of papaya fruit ripening and to reduce the postharvest losses.

In this study, the expressions of ethylene biosynthesis, receptors, and response genes was examined during fruit ripening. The expression of ethylene biosynthesis related enzymes, CpACS and CpACO, are expected to increase in the early fruit maturation stage. Based on previous studies, the ethylene receptors may be involved in the initiation of fruit ripening. At least one of the ethylene receptors is expected to be expressed, and

the expression should increase along with massive ethylene production at the onset of fruit ripening. Furthermore, CpETR may affect CpCTR1 expression level, since the CTR1 kinase forms a complex with ethylene receptor on the endoplasmic reticulum membrane and acts as a negative regulator in ethylene signal transduction pathway. Transcriptional factors, EIN2, EIN3/EIL, and ERFs, may also show differential expression during fruit development, ripening and maturation.

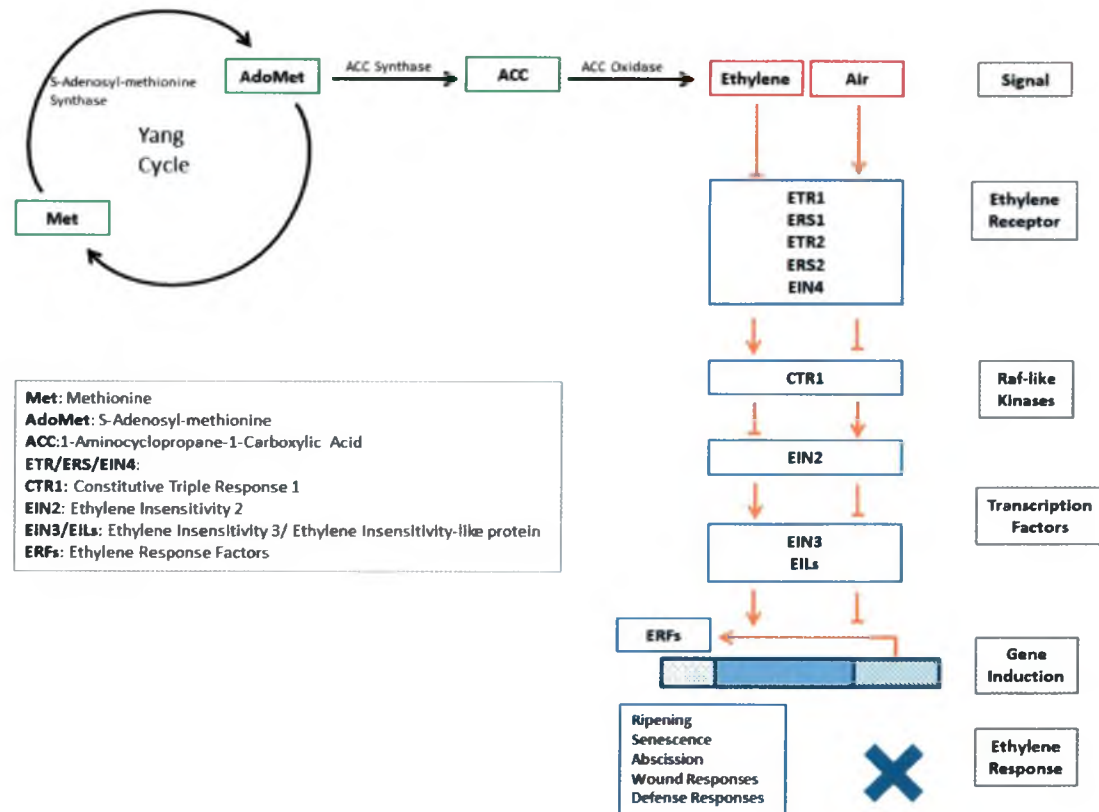


Figure 1 Representation of the ethylene biosynthesis and signal transduction pathway



## **2. Methods and data analysis**

### **2.1 Plant Material and Fruit Quality**

Papaya SunUp fruit were harvested at the mature green to color break stage, and allowed to ripen at room temperature (about 22°C). Fruit weight, respiration rate and ethylene production were measured during ripening of each fruit. Fruit was sampled at four stages of fruit ripening based on degree of skin yellowing: mature green, 25-30%, 75-80% and 100% yellowing. Ethylene production was detected at the 75-80% yellow stage. Three fruit from each ripening stage were sampled, and a composite mesocarp sample was made by combining the three random fruit.

### **2.2 Fruit Firmness Determination, Respiration Rate and Ethylene Production**

The papaya fruit firmness was determined using a penetrometer (LKG-14, Amtek, Largo, FL). The force depressing a 1.5 cm diameter disc 1mm into the fruit was recorded.

Fruit was enclosed in a sealed ca. 2,500 mL plastic container at room temperature. After one hour, 1mL of headspace gas was sampled with a syringe and injected it into a carbon dioxide analyzer (Model LI-6251 Infrared Gas Analyzer, LI-COR Inc., Lincoln, NE) for respiration rate determination and another 1mL into a gas chromatograph (Shimazu Model GC-8A, Shimazu Corp., Kyoto, Japan) for ethylene production determination.

### **2.3 Total RNA Isolation**

Total RNA is extracted from the papaya mesocarp of each stage using with phenol/chloroform extraction method (Mason and Botella, 1997). The fruit was ground into powder in liquid nitrogen and the powder mixed with 3 volumes of extraction buffer (150mM Tris base, 2% (w/v) sodium dodecyl sulfate, 50mM Ethylenediaminetetraacetic acid, 1%  $\beta$ -mercaptoethanol, adjust to pH 7.5). Absolute ethanol was added to 0.25 volumes plus 0.11X of 5 M potassium acetate, and stirred for two minutes. Add equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) were added to the tube and shaken for five minutes at room temperature. The homogenate was centrifuged at 12,500 rpm for 50 minutes at 4°C. Total RNA was extracted from pellet with an equal volumes of phenol-chloroform-isoamyl alcohol (25:24:1) until no interphase layer appear and precipitated with 2.25 volume of absolute ethanol at -20°C overnight. After centrifugation at 12,000 rpm for 40 minutes at 4°C, the pellet was washed several times with 70% ethanol. The pellet was resuspended in DEPC-treated water, then precipitate with 12M lithium chloride at -20°C overnight, centrifuged at 12,000 rpm for 40 minutes at 4°C. The pellet was again washed with 80% ethanol and resuspended in DEPC-treated water. The total RNA quality were analyzed on NanoDrop Spectrophotometer (ND-1000, Thermo Fisher Scientific Inc., Rockford, IL), and both the A260/A280 and A260/A230 ratios were greater than 1.8.

### **2.4 mRNA Purification and cDNA Synthesis**

A magnetic mRNA purification kit (Poly-A Purist MAG, Ambion Inc., Austin, TX) was used to isolate mRNA from the total RNA. Purified mRNA was reversed transcribed

into cDNA using the SuperScript Double-Stranded cDNA Synthesis Kit (Invitrogen, Carlsbad, CA).

## **2.5 Gene Expression and cDNA Microarray**

The cDNA samples were labeled using the NimbleGen One Color DNA Labeling Kit (Roche NimbleGen, Madison, WI). The Cy3-labeled samples were hybridized to the NimbleGen (Roche NimbleGen, Madison, WI) papaya arrays. The 4x72k papaya arrays were made using a maskless photolithography oligonucleotide (60 mer) synthesis process, and have 24,421 predicted genes that were represented by two to three probes within each quadrant. No unique probes could be designed for 353 predicted sequences, so these were not represented. Seventeen probes detect more than one sequence.

Scanning the arrays produced the images of sequences with data extraction software to select the genes with different images and determine the expression levels of ethylene receptors during fruit ripening. The hybridization array was scanned at 532nm with the GenePix 4100A microarray scanner (Molecular Devices, Inc., Sunnyvale, CA), and the spot intensities were analyzed using GenePix Pro version 6.1. The data background correction, local normalization, and summarization were done with the NimbleScan software (Roche NimbleGen, Madison, WI).

The preprocessed data from NimbleScan software were analyzed by general linear model to test the significant differences of gene expression levels on log<sub>2</sub> scale among stages and genes. The distribution of gene expression levels were examined by Q-Q plots and skewness values through the Proc univariate procedure in SAS 9.1.3 (Cary, NC).

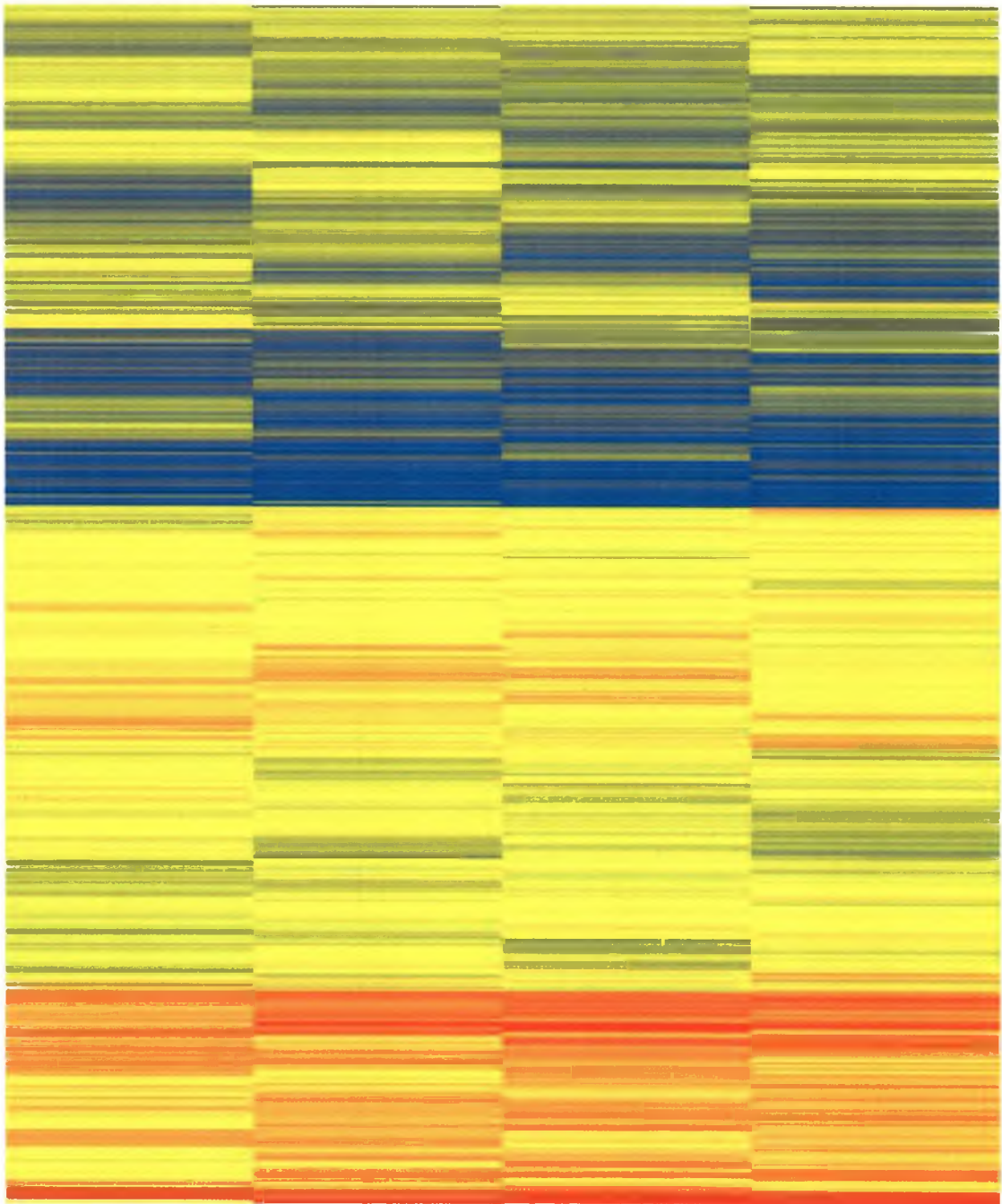
Tukey's multiple comparison procedures were used for pairwise comparisons among stages for each gene. Dunnett's tests were used for comparing the differences between each ripening stage with the mature green stage. The P-values for each gene were adjusted to control the familywise error rate by the Bonferroni procedure.

To analyze multiple slides, RMA (Robust Multi-array Analysis) background correction and quantile normalization were done in ArrayStar (DNASTAR Inc., WI) software. All gene expression levels from three biological replicates should be distributed identically across arrays. The data processing was followed by the analysis of variance (ANOVA) with p-value adjusted by the Benjamini-Hochberg procedure to control the false discovery rate (FDR) at 5% in ArrayStar software.

### **3. Results**

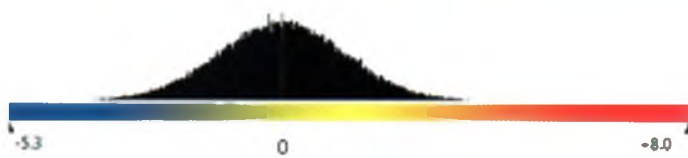
#### **3.1 The Papaya Genome at Four Different Maturation Stages**

The Papaya genome was predicted to contain 24,744 genes. The papaya gene expression level on heatmap was ranged from 2.9 to 14.5 on log2 scale. The heatmap with the color scale based on the gene expression levels at green mature stage showed that most of the genes in papaya are down-regulated during fruit ripenings. The expression levels of genes at different stages were compared using the F test in ANOVA with FDR adjustment at 5%. In the ArrayStar default ANOVA testing, only 24,410 genes had valid values, and the genes with the adjusted p value less than 0.05 were selected. Based on the analysis, none of the genes show significant differences between maturation stages.



**Figure 2 Heatmap of the expression all papaya genes in papaya fruit**

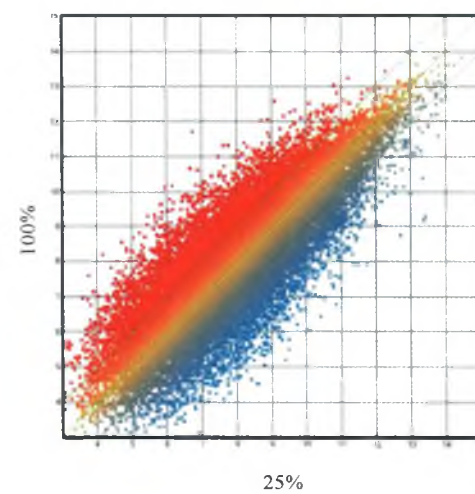
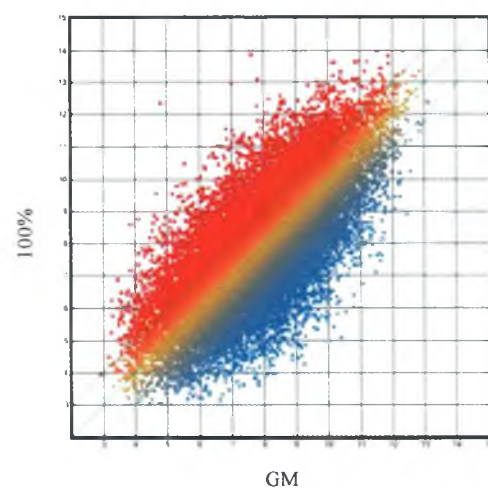
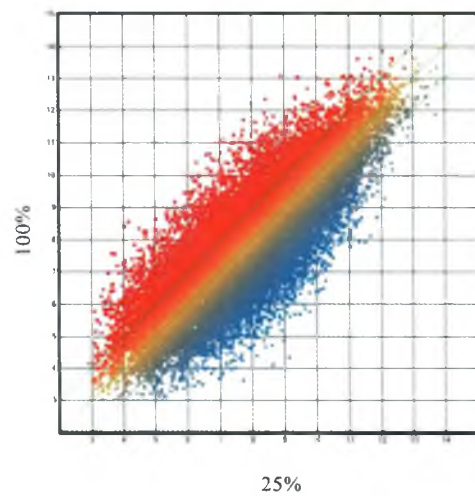
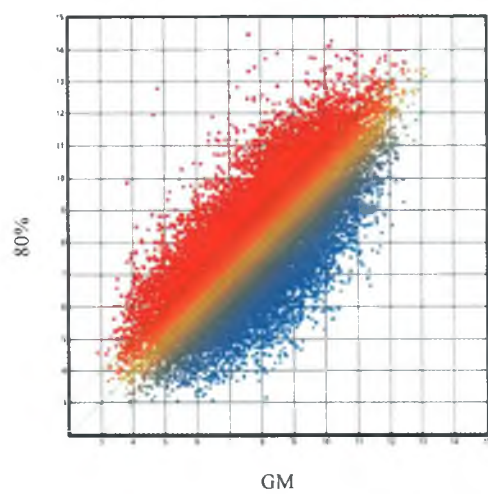
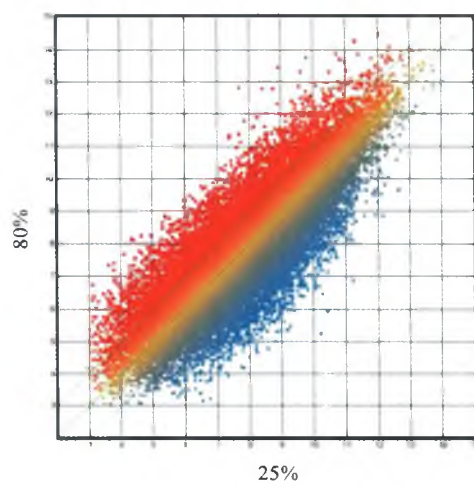
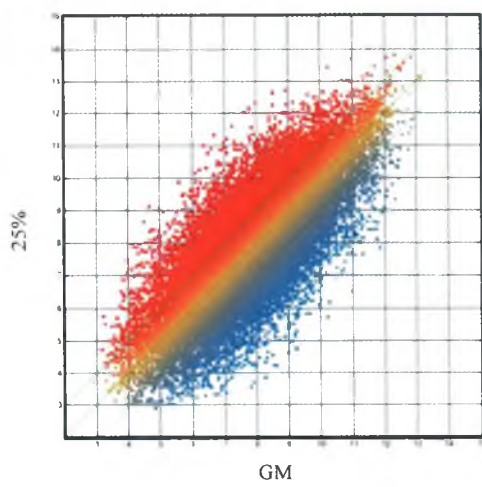
The papaya genes expressed at different maturation stage: mature green, 25-30% color, 75-80% color, and 100% color were clustered. The clustering was done in ArrayStar (DNASTAR, Inc, Madison, Wisconsin, USA) using Hierarchical clustering method based on Euclidean distance metric. This heatmap used colors to represent the relative value of expressed genes within giving conditions. Blue color indicates low expression, yellow indicates intermediate expression and red color represents a relative high expression. The numerical values represent the actual gene expression levels on a log 2 scale and which were associated with each color. The color scale was shown as a bar below of the figure.





### **Figure3 Heatmap of the expression all papaya genes in papaya fruit**

The papaya genes expressed at different maturation stage: mature green, 25-30% color, 75-80% color, and 100% color were clustered. The clustering was done in ArrayStar (DNASTAR, Inc, Madison, Wisconsin, USA) using Hierarchical clustering method based on Euclidean distance metric. This heat map used per-row color scaling which was used an independent color scale for each row. The values for each gene were displayed as the difference between each column and the experiment baseline (mature green stage). The color scale was shown as a bar below the figure and the dotted line in histogram above the color scale indicated the zero point for baseline experiment.



**Figure 4     Scatter plots for the comparison of gene expression levels between different stages**

Three solid green lines were drawn diagonally across the scatter plot. The middle green line was the identity line ( $x=y$ ), and the other two lines delineate genes with at least a two-fold change in intensity value in one of the data sets. Each data point represented an individual gene based on its expression level in both selected stages.

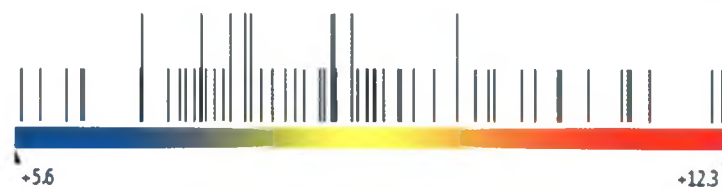
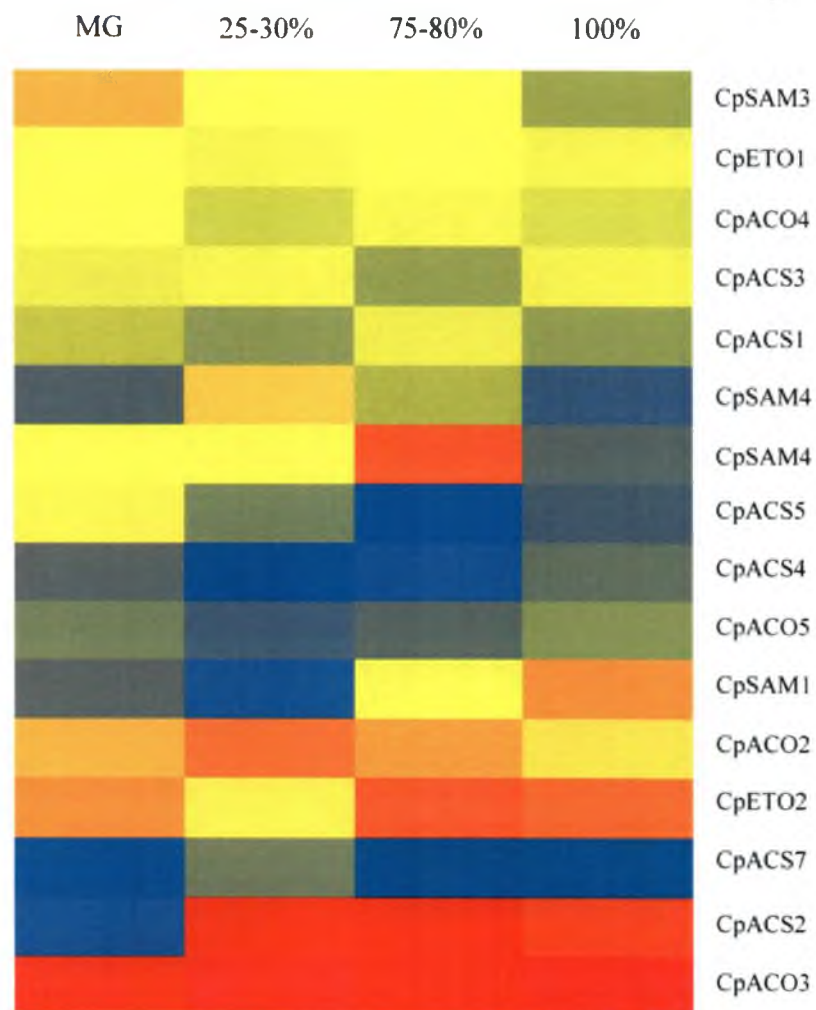
### 3.2 Ethylene Biosynthesis Gene Expression in Papaya

The ethylene biosynthesis genes showed differential expression patterns during fruit ripening. The highest expressed level of CpSAM3 occurred at the mature green stage, and then declined throughout fruit ripening (Figure 5, 7). CpSAM4 was expressed at the same level as CpSAM3 at the beginning, but then showed a 4-fold increase between the 25-30% color and 75-80% color stage (Figure 7, 8). The highest expression of CpSAM4 appeared at the stage with 75-80% yellow skin, and then declined to its lowest expression level when the fruit was fully ripe (Figure 7). Unlike the down-regulation of CpSAM3, CpSAM1 was up-regulated during fruit ripening, though it initially declined during early ripening (Figure 6).

Two different patterns of CpACS gene expression were found (Figure 5, 9). The first pattern included three CpACS homologs, CpACS1, CpACS2, and CpACS7. All these showed an increase in expression level at the 25-30% yellow stage. At the 75-80% stage, CpACS1 and CpACS7 were down-regulated, while CpACS2 was highly expressed until the fruit reaches the full ripen stage (Figure 10). The second pattern was shown by the three CpACS homologs, CpACS3, CpACS4 and CpACS5 (Figure 9). All were down-regulated from mature green to 25-30% color stage (Figure 10). The expression of CpACS4 and CpACS5 reached the lowest level of expression at the 25-30% and 75-80% yellow stage, respectively (Figure 5, 9). Both gene expressions increased when the fruit was fully ripe. CpACS3 was up-regulated at the 75-80% yellow skin stage (Figure 10), but the level of gene expression declined to the same level found at mature green stage (Figure 9).

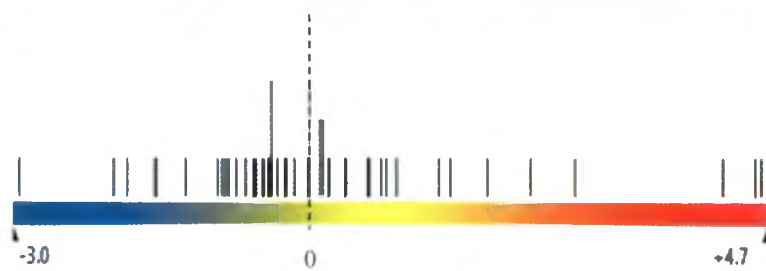
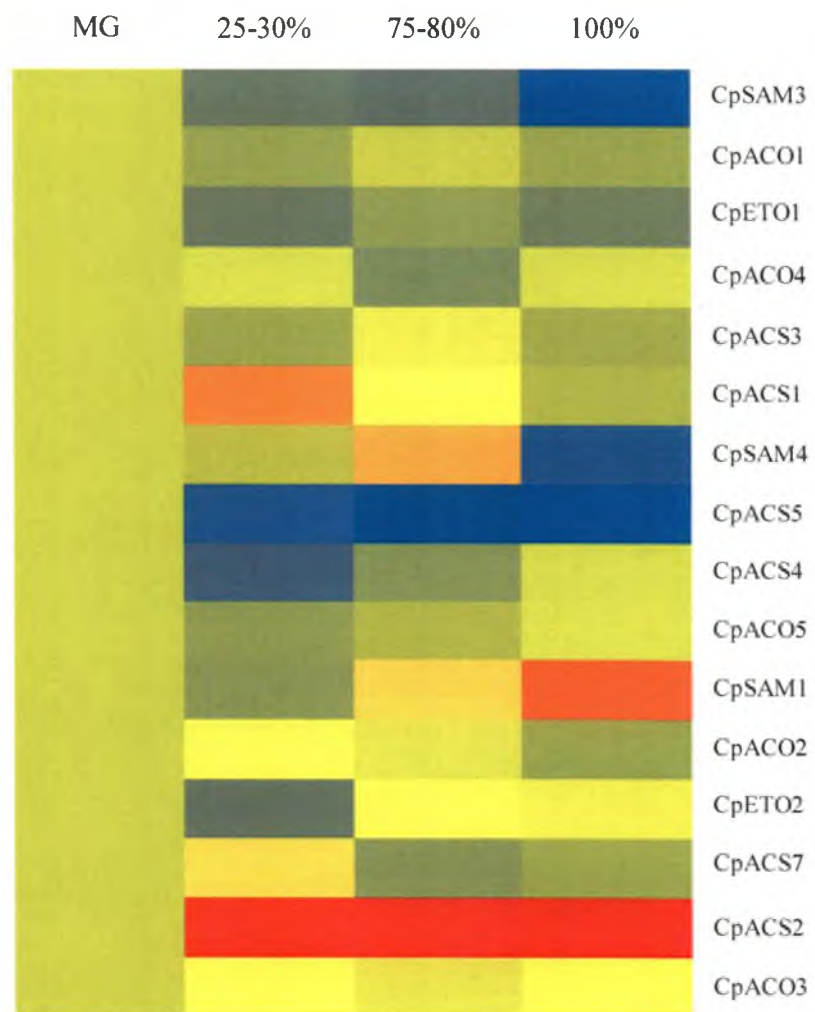
Unlike the CpACS, CpACO homologs did not show any obvious changes during fruit ripening. However, they were generally up-regulated during early fruit ripening (Figures 6, 11).

The two ethylene biosynthesis genes that function as ETO (Ethylene-over-producer) were both up-regulated from mature green to the 75-80% yellowing stage, and the expression levels of both genes then declined after the full ripen stage (Figure 13).



**Figure 5 Heatmap of the expression of ethylene biosynthesis genes in papaya fruit mesocarp during ripening**

CpSAM, CpACS, CpACO, and CpETO genes at different maturation stages: mature green, 25-30% color, 75-80% color, and 100% color were clustered. The clustering was done by ArrayStar (DNASTAR, Inc, Madison, Wisconsin, USA) with Hierarchical clustering method based on Euclidean distance metric (linkage method: centroid). This heatmap used colors to represent the relative value of expressed genes within the giving condition. Blue color indicates low expression, yellow indicates intermediate expression and red color represents a relative high expression. The numerical values given were the actual values on a log 2 scale and were associated with each color. The color scale shown as a bar below the figure.

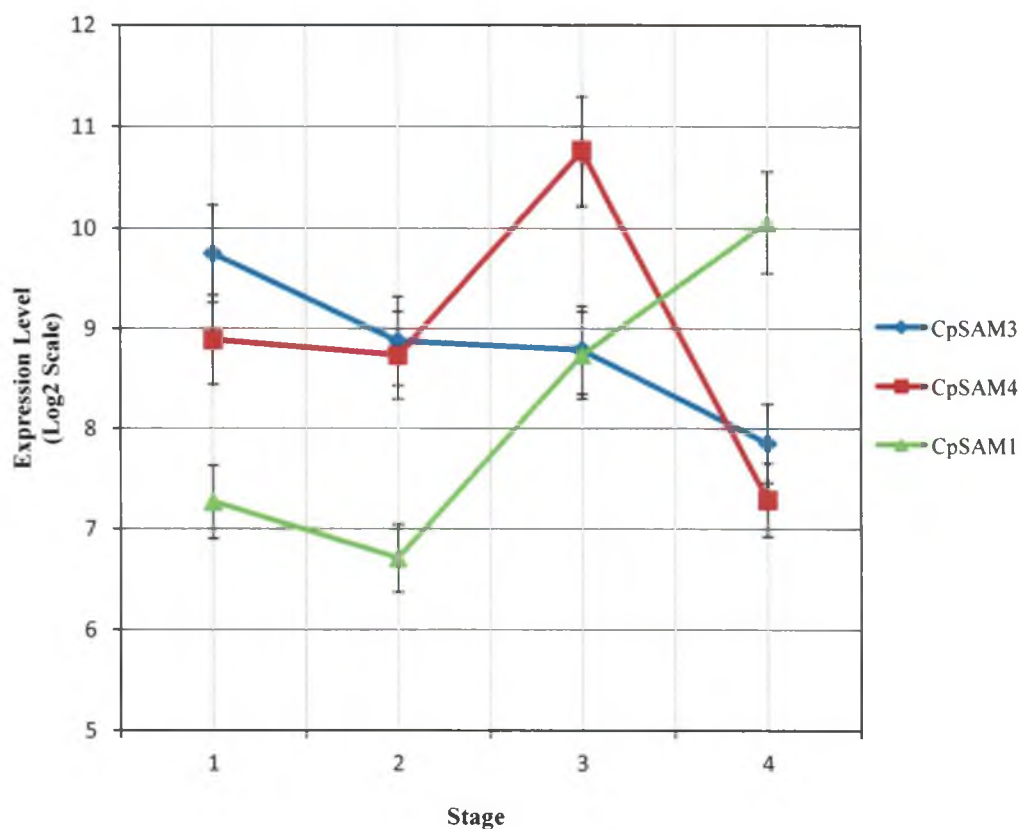




**Figure 6 Heatmap of the expression of ethylene biosynthesis genes in papaya fruit mesocarp during ripening**

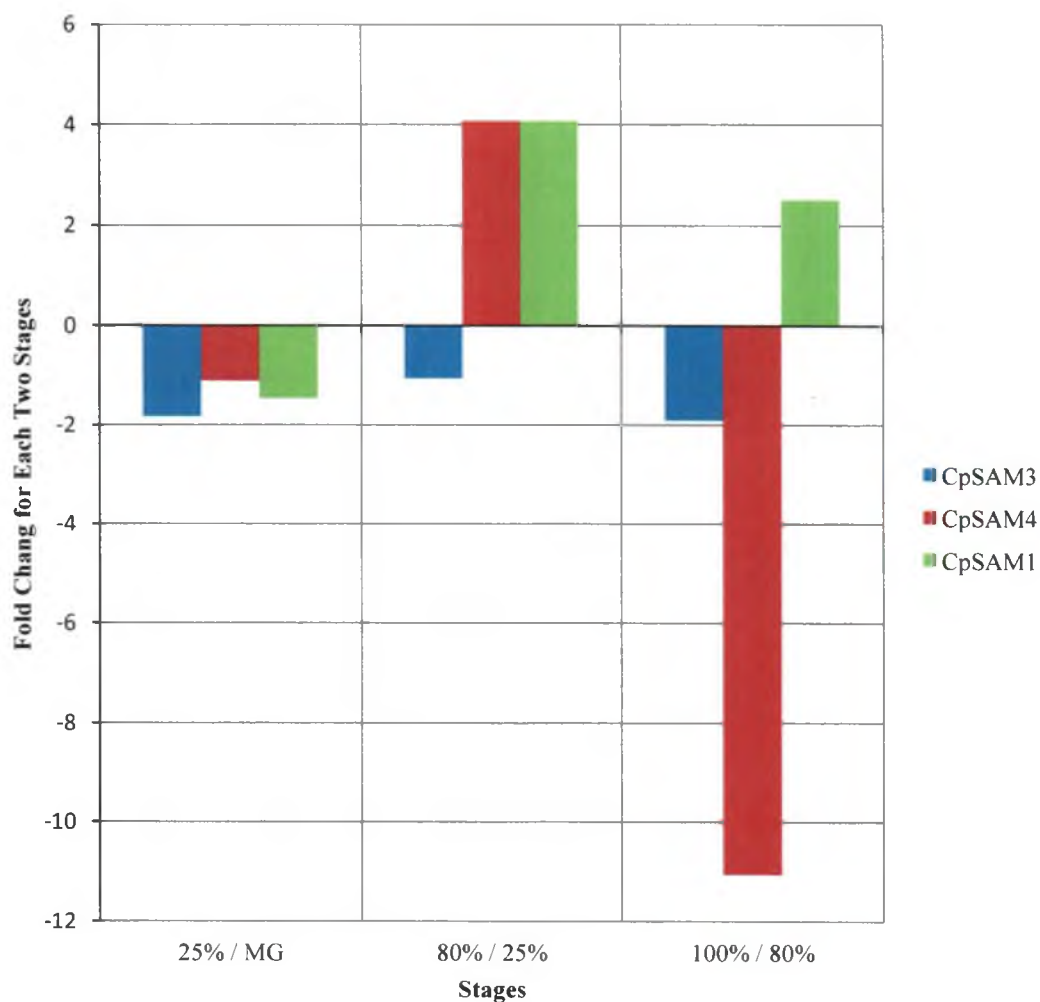
CpSAM, CpACS, CpACO, and CpETO genes at different maturation stages: mature green, 25-30% color, 75-80% color, and 100% color were clustered. The clustering was done by ArrayStar (DNASTAR, Inc, Madison, Wisconsin, USA) with Hierarchical clustering method based on Euclidean distance metric (linkage method: centroid). This heatmap used per-row color scaling with an independent color scale for each row. The values for each gene were displayed as the difference between each column and the experiment baseline (mature green stage). The color scale shown as a bar below the figure and the dot line in histogram above the color scale indicates the zero point for baseline comparison.

## Papaya Biosynthesis Genes -CpSAM-



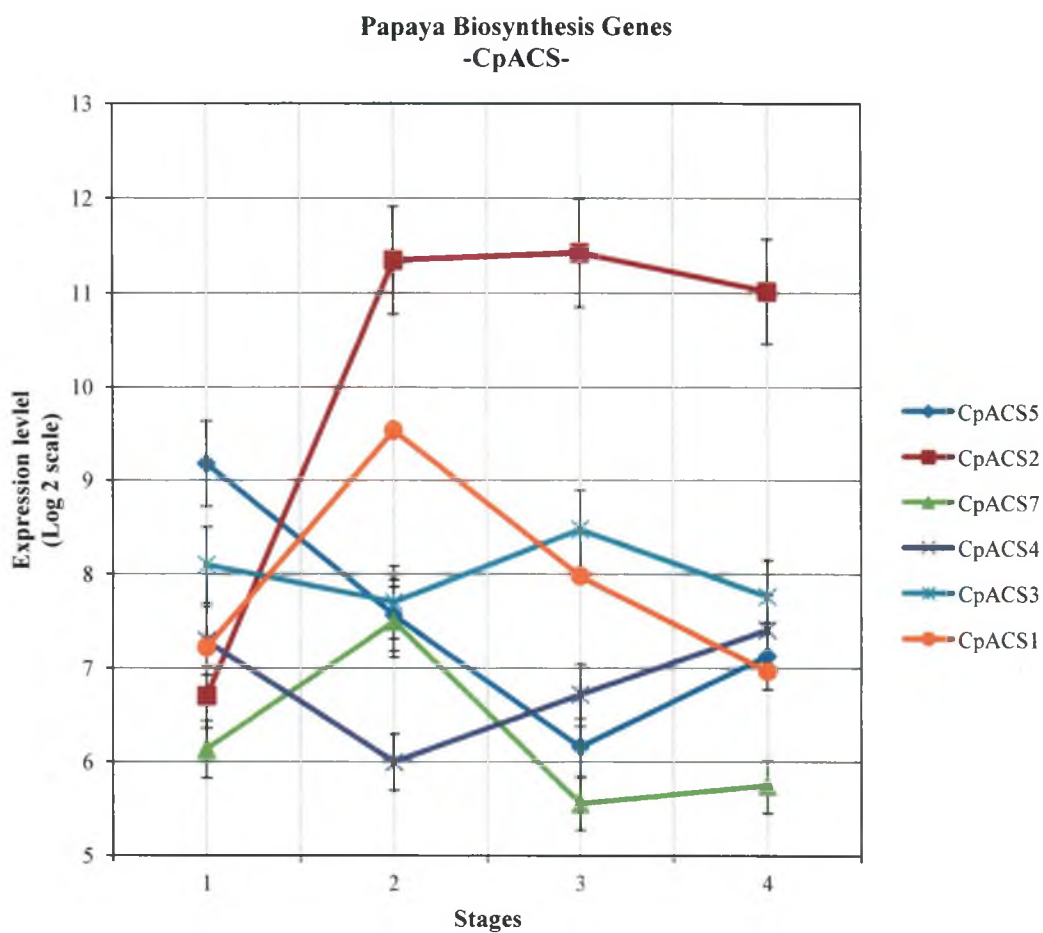
**Figure 7** Expressions of ethylene biosynthesis genes: CpSAM (SAM Synthase; S adenosylmethionine synthase) in papaya fruit mesocarp during ripening

Stages 1, 2, 3, and 4 in x axis indicated mature green (1), 25-30% (2), 75-80% (3) and 100% (4) color stages, respectively. Y axis shows the expression on a log 2 scale (maximum value: 16).



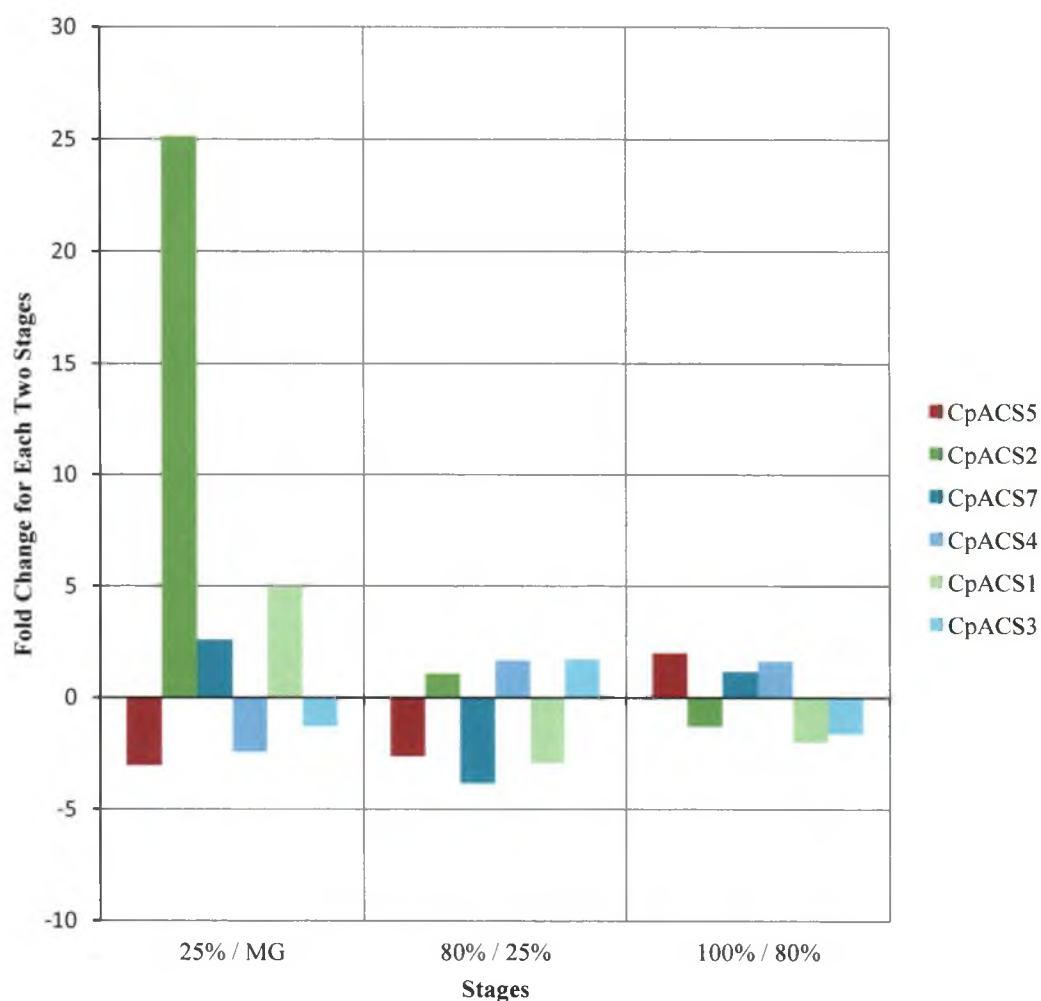
**Figure 8 The fold-change of CpSAM (SAM Synthase) gene expressions between each two stages**

The “25% / MG” on x axis represented the comparisons of the gene expression at 25% color stage and that in mature green stage (MG). The “80% / 25%” on x axis indicated the comparisons of the gene expression at 80% color stage and that at 25% color stage. The “100% / 80%” on x axis was the comparisons of the gene expression at 100% color stage and that at 80% color stage. The two-stage comparison was estimated gene expression levels on log 2 scale from liner models.



**Figure 9** Expressions of ethylene biosynthesis genes: CpACS (ACC Synthase, 1-aminocyclopropane-1-carboxylate synthase) in papaya fruit mesocarp during ripening

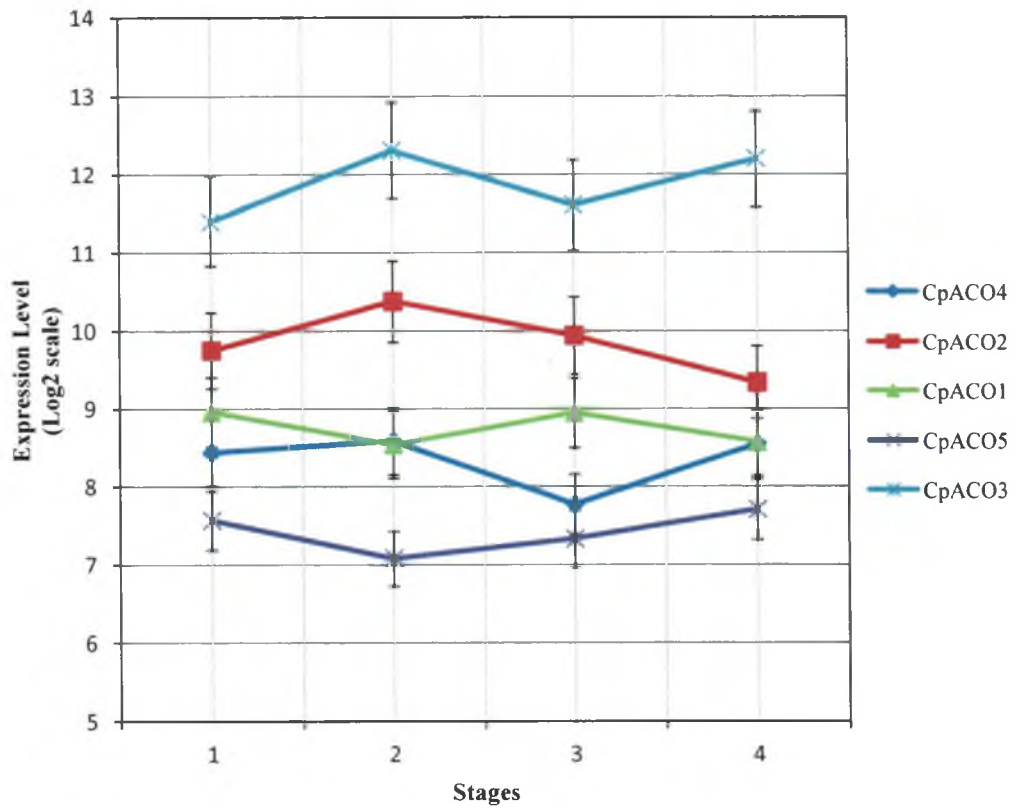
Stages 1, 2, 3, and 4 in x axis indicated mature green (1), 25-30% (2), 75-80% (3) and 100% (4) color stages, respectively. Y axis shows the expression on a log 2 scale (maximum value: 16).



**Figure 10 The fold-change of CpACS (ACC synthase) gene expressions between each two stages**

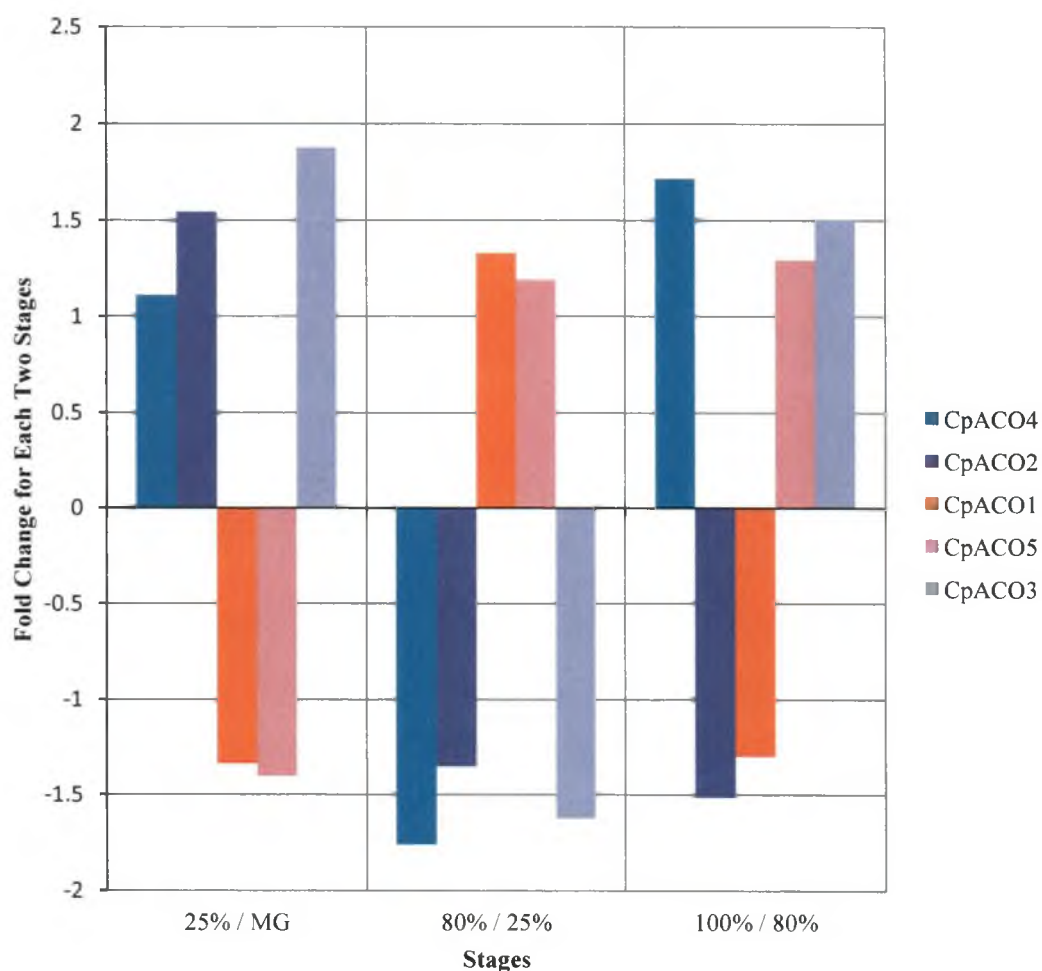
The “25% / MG” on x axis represented the comparisons of the gene expression at 25% color stage and that in mature green stage (MG). The “80% / 25%” on x axis indicated the comparisons of the gene expression at 80% color stage and that at 25% color stage. The “100% / 80%” on x axis was the comparisons of the gene expression at 100% color stage and that at 80% color stage. The two-stage comparison was estimated gene expression levels on log 2 scale from liner models.

### Papaya Biosynthesis Genes -CpACO-



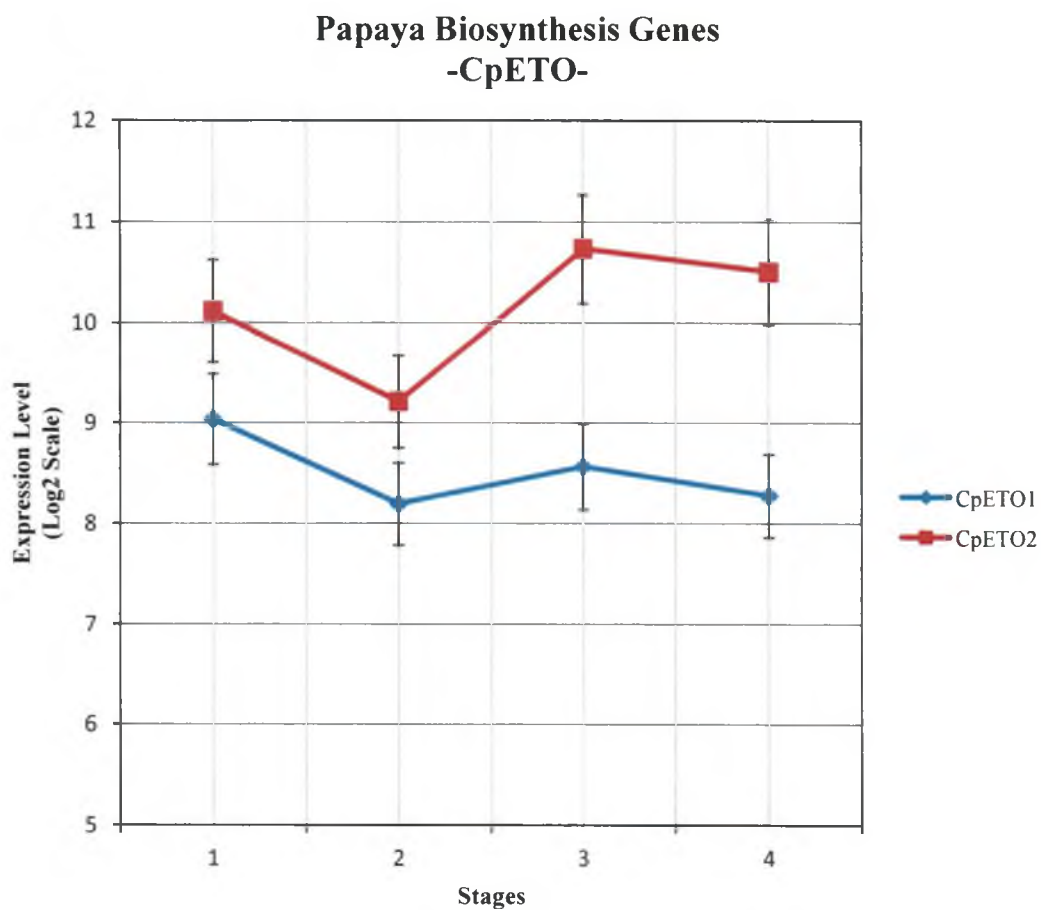
**Figure 11** Expressions of ethylene biosynthesis genes CpACO (ACC oxidase, 1-aminocyclopropane-1-carboxylate oxidase) in papaya fruit mesocarp during ripening

Stages 1, 2, 3, and 4 in x axis indicated mature green (1), 25-30% (2), 75-80% (3) and 100% (4) color stages, respectively. Y axis shows the expression on a log 2 scale (maximum value: 16).



**Figure 12** The fold-change of CpACO gene expressions between each two stages

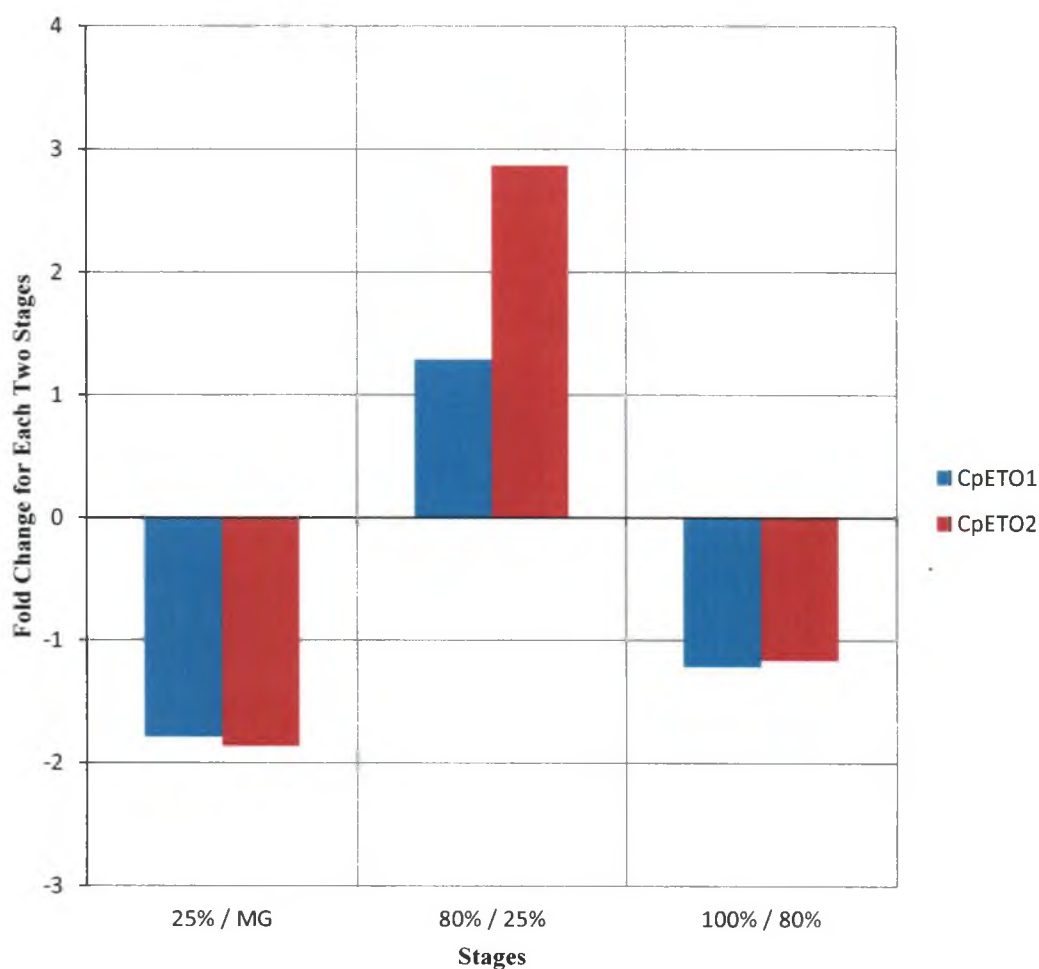
The “25% / MG” on x axis represented the comparisons of the gene expression at 25% color stage and that in mature green stage (MG). The “80% / 25%” on x axis indicated the comparisons of the gene expression at 80% color stage and that at 25% color stage. The “100% / 80%” on x axis was the comparisons of the gene expression at 100% color stage and that at 80% color stage. The two-stage comparison was estimated gene expression levels on log 2 scale from liner models.



**Figure 13** Expressions of ethylene biosynthesis genes CpETO (Ethylene over-producer) in papaya fruit mesocarp during ripening

Stages 1, 2, 3, and 4 in x axis indicated mature green (1), 25-30% (2), 75-80% (3) and 100% (4) color stages, respectively. Y axis shoes the expression on a log 2 scale (maximum value: 16).



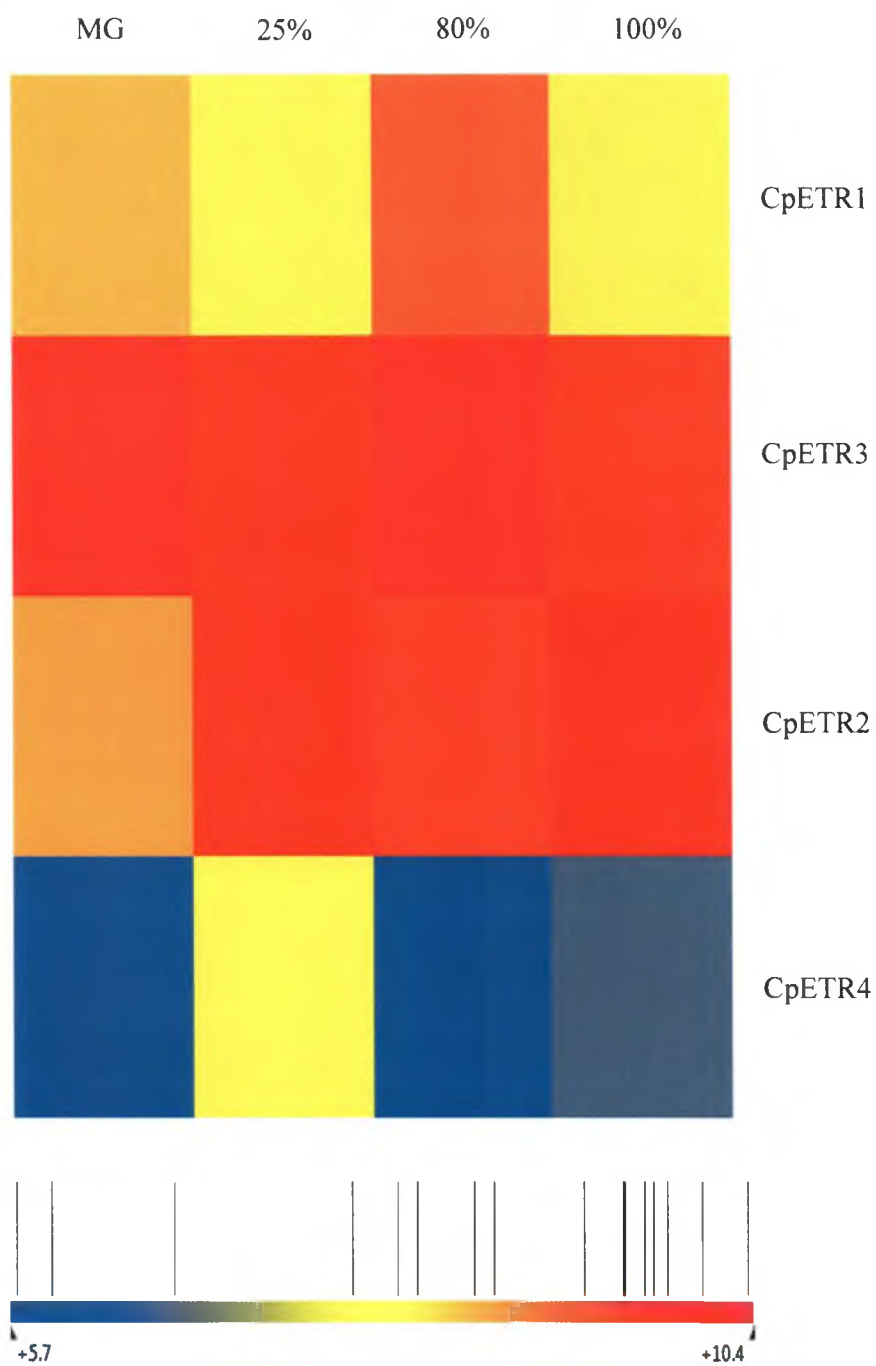


**Figure 14 The fold-change of CpETO (Ethylene overproducer) gene expressions between each two stages**

The “25% / MG” on x axis represented the comparisons of the gene expression at 25% color stage and that in mature green stage (MG). The “80% / 25%” on x axis indicated the comparisons of the gene expression at 80% color stage and that at 25% color stage. The “100% / 80%” on x axis was the comparisons of the gene expression at 100% color stage and that at 80% color stage. The two-stage comparison was estimated gene expression levels on log 2 scale from liner models.

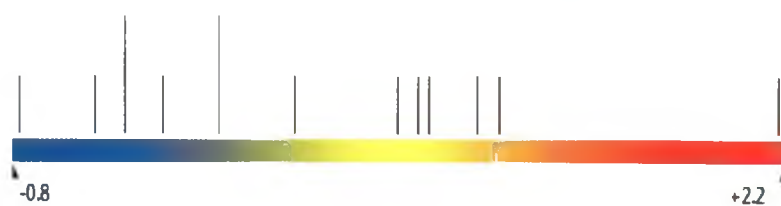
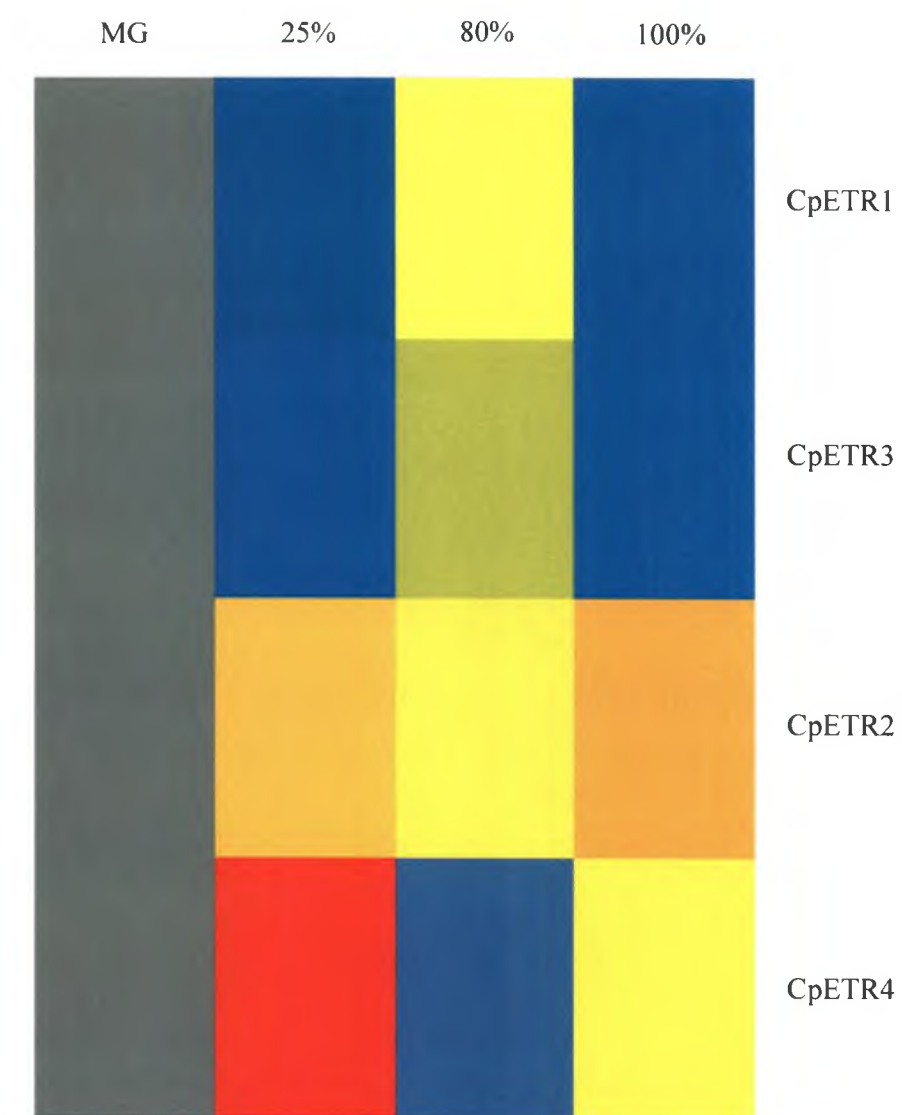
### **3.3 Expressions of Ethylene Perception Genes in Papaya Fruit**

During early ripening (from mature green to 25-30% color stage), CpETR1 and CpETR3 were down-regulated, and CpETR2 and CpETR4 were up-regulated (Figure 18). The expression of CpETR4 increased during early ripening, and maintained a similar expression level until the full ripe stage (Figure 17). Unlike CpETR4, CpETR2 showed a 4-fold up-regulation at 25-30% yellow skin stage, and then was down-regulated from the 25-30% color to 75-80% color stage. During fruit ripening, the gene expressions of the three ethylene receptor, CpETR1, CpETR2, and CpETR3, were either up-regulated or down-regulated before the full ripe stage when they returned to the same level as that of the mature green stage (Figure 16, 17). Only CpETR4 showed general up-regulation during fruit ripening (Figure 15, 17).



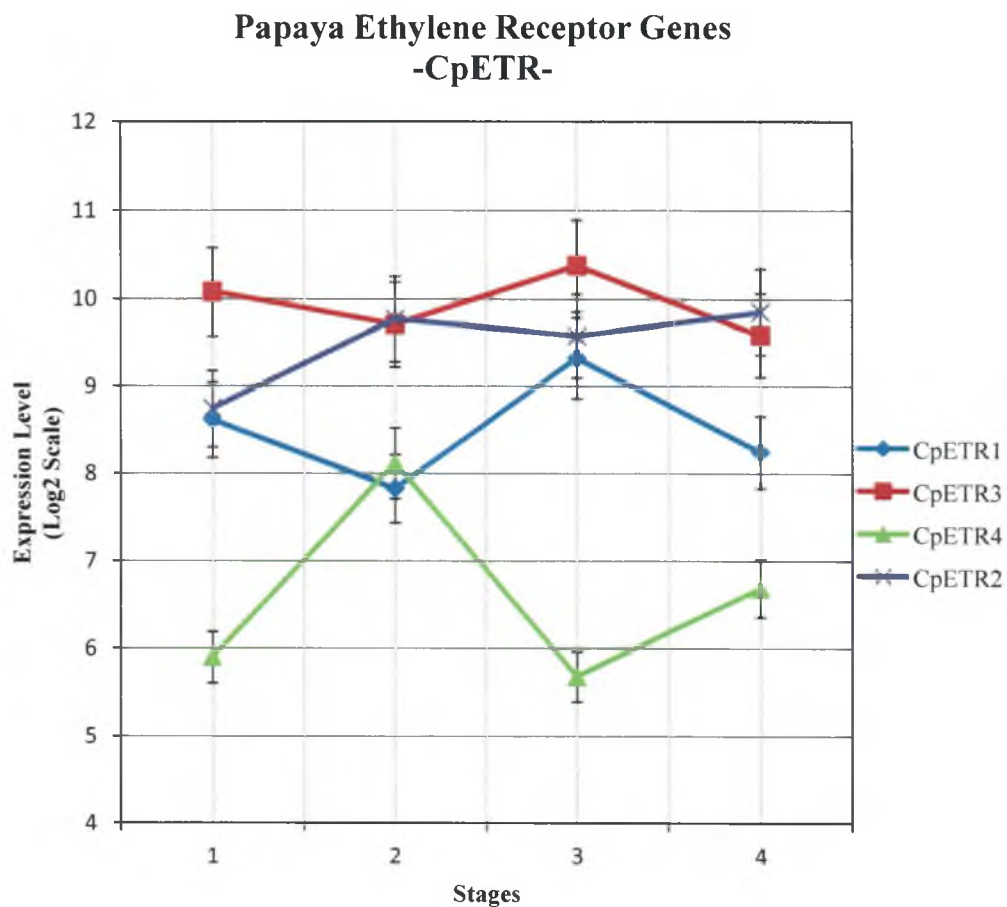
**Figure 15 Heatmap of the expression of four ethylene receptor genes in papaya fruit mesocarp during fruit ripening**

The four papaya ethylene receptor (CpETR 1 to 4) genes at different maturation stages: mature green, 25-30% color, 75-80% color, and 100% color were clustered. The clustering was done by ArrayStar (DNASTAR, Inc, Madison, Wisconsin, USA) with Hierarchical clustering method based on Euclidean distance metric (linkage method: centroid). This heat map used colors to represent the relative value of expressed genes within the giving condition. Blue color indicates low expression, yellow indicates intermediate expression and red color represents a relative high expression. The numerical values given were the actual values on a log 2 scale and were associated with each color. The color scale is shown as a bar below the figure.



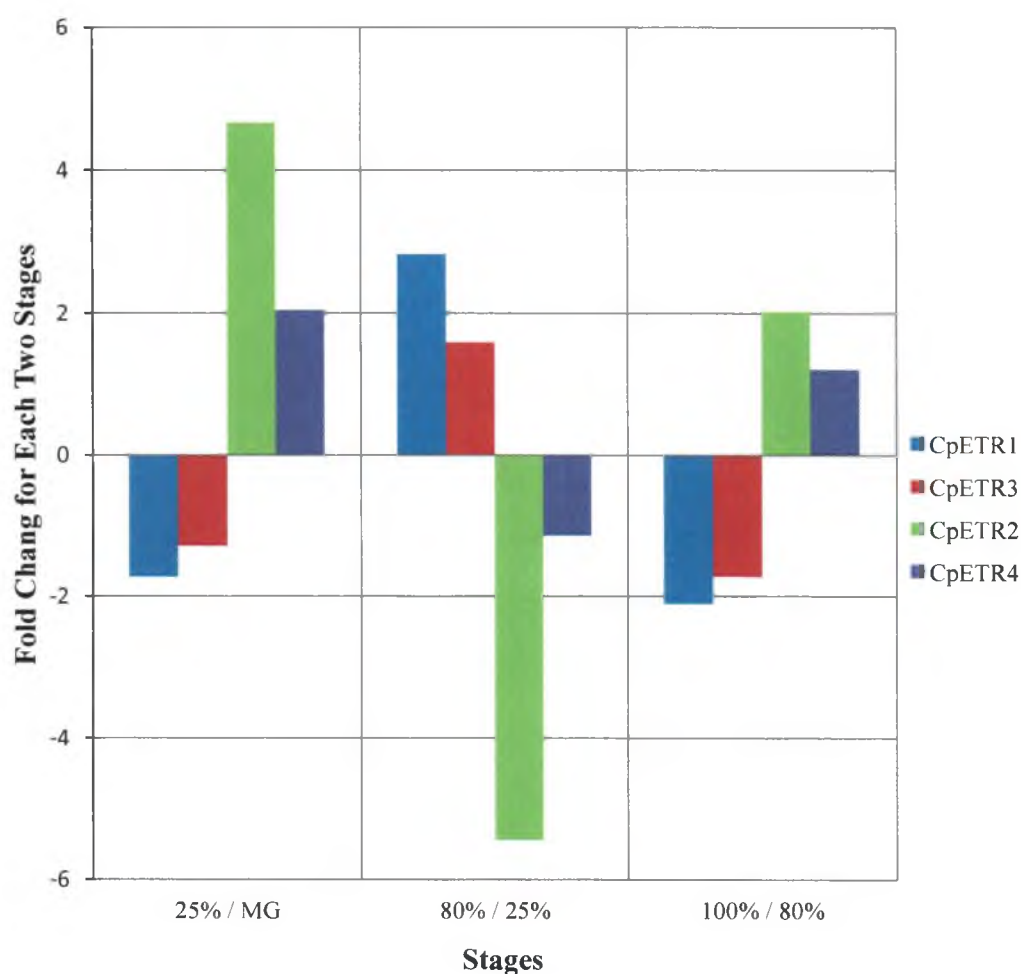
**Figure 16 Heat Map of the expression of four ethylene receptor genes in papaya fruit mesocarp during fruit ripening**

The four papaya ethylene receptor (CpETR1 to 4) genes at different maturation stages: mature green, 25-30%, 75-80%, and 100% color were clustered. The clustering was done using ArrayStar (DNASTAR, Inc, Madison, Wisconsin, USA) with Hierarchical clustering method based on Euclidean distance metric (linkage method: centroid). This heat map used per-row color scaling that used an independent color scale for each row. The values for each gene were displayed as the difference between each column and the experiment baseline (mature green stage). The color scale is shown as a bar below the figure and the dot line in histogram above the color scale indicates the zero point for baseline comparison.



**Figure 17** Expressions of four ethylene receptor genes (CpETR1-4) in papaya fruit mesocarp during ripening

Stages 1, 2, 3, and 4 on x axis indicated mature green (1), 25-30% (2), 75-80% (3) and 100% (4) color stages, respectively. Y axis shoes the expression on a log 2 scale (maximum value: 16).



**Figure 18 The fold-change of ethylene receptor gene expressions between each two stages**

The “25% / MG” on x axis represented the comparisons of the gene expression at 25% color stage and that in mature green stage (MG). The “80% / 25%” on x axis indicated the comparisons of the gene expression at 80% color stage and that at 25% color stage. The “100% / 80%” on x axis was the comparisons of the gene expression at 100% color stage and that at 80% color stage. The two-stage comparison was estimated gene expression levels on log 2 scale from liner models.

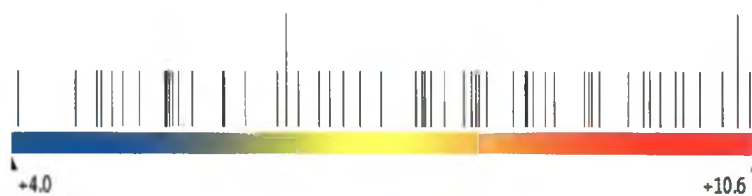
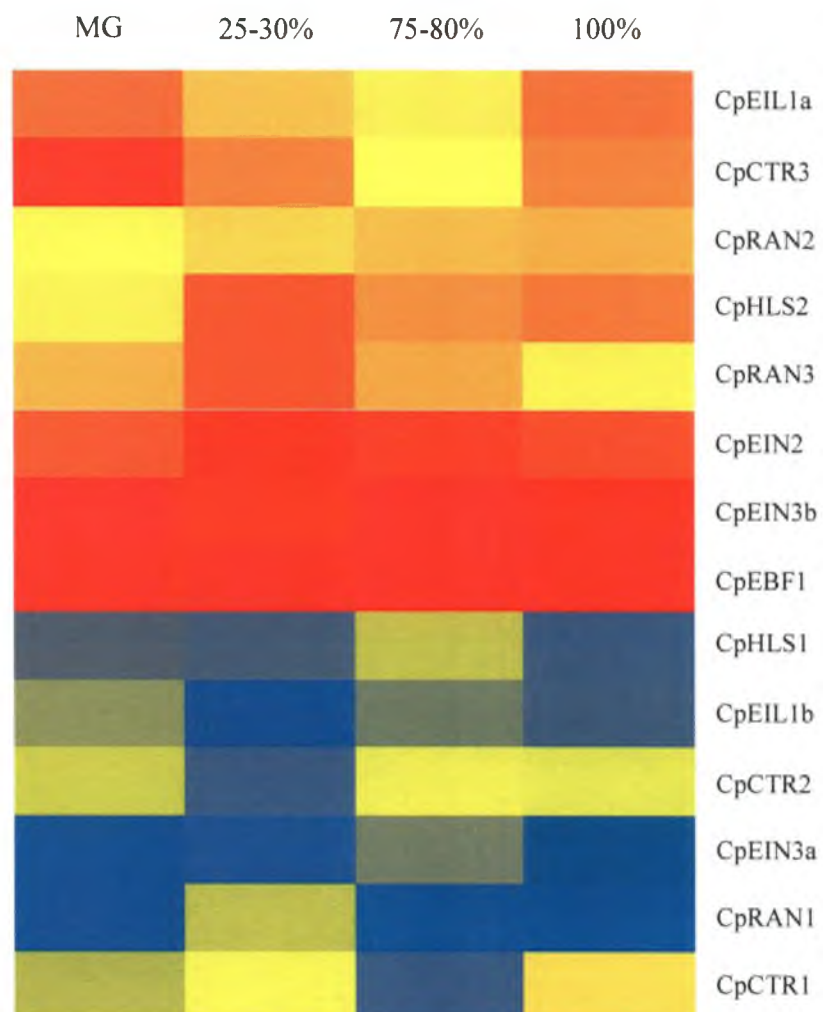


### 3.4 Expressions of Ethylene Response Genes in Papaya Fruit

Both CpRAN1 and CpRAN3 were up-regulated during early ripening (Figure 20), and then declined after the highest expression level at the 25-30% color stage (Figure 21). Unlike CpRAN1 and CpRAN3, CpRAN2 expression increased throughout fruit ripening (Figure 21).

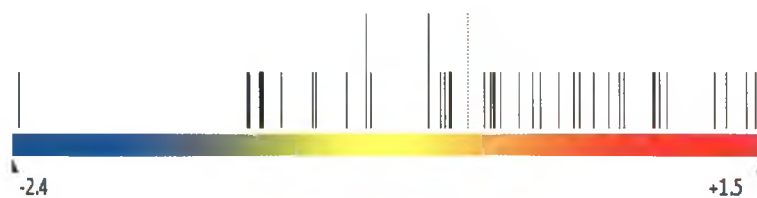
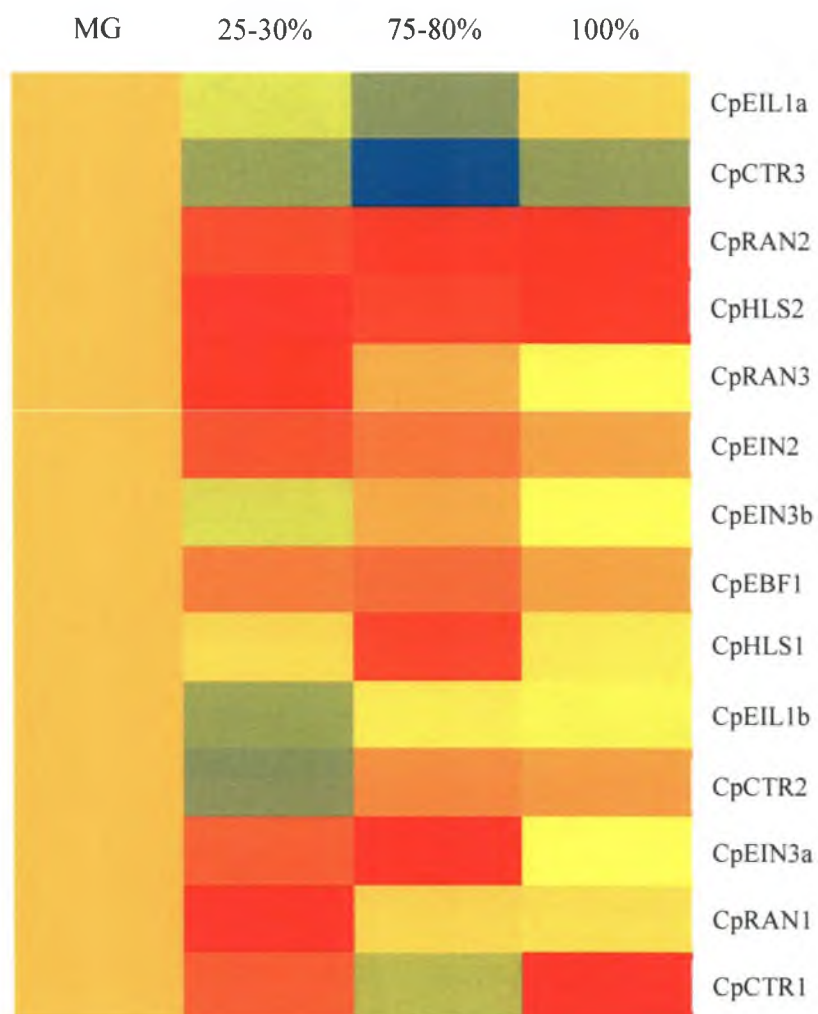
CpCTR1 and CpCTR2 were overall down-regulated during papaya fruit ripening (Figure 19). The difference between CpCTR1 and CpCTR2 was in the timing when the lowest expression level occurred (Figure 20, 23). The lowest expression level of CpCTR1 occurred at the 75-80% color stage, which was later than that of CpCTR2 (Figure 23). CpCTR3 was down-regulated during fruit ripening and showed the highest expression level at mature green stage, and then declined at the 25% color and 80% color stages (Figure 24). Similar to the expression pattern of CpCTR1, the expression level of CpCTR3 at 100% color stage was lower than that at the mature green stage (Figure 23).

CpEIN2 was up-regulated from the mature green to the 25-30% color stage (Figure 26), and remained of that expression level until the full ripe stage (Figure 20). Only one EIN3-like gene was down-regulated during early ripening (Figure 25). The remainder of the EIN3, EIN3-like and EBF genes were generally up-regulated throughout fruit ripening (Figure 19, 20), and the highest expression level of each gene occurred at different stages vary from the 25-30% to fully ripen stage (Figure 25).



**Figure 19 Heat Map of the expression of ethylene signaling genes in papaya fruit mesocarp during fruit ripening**

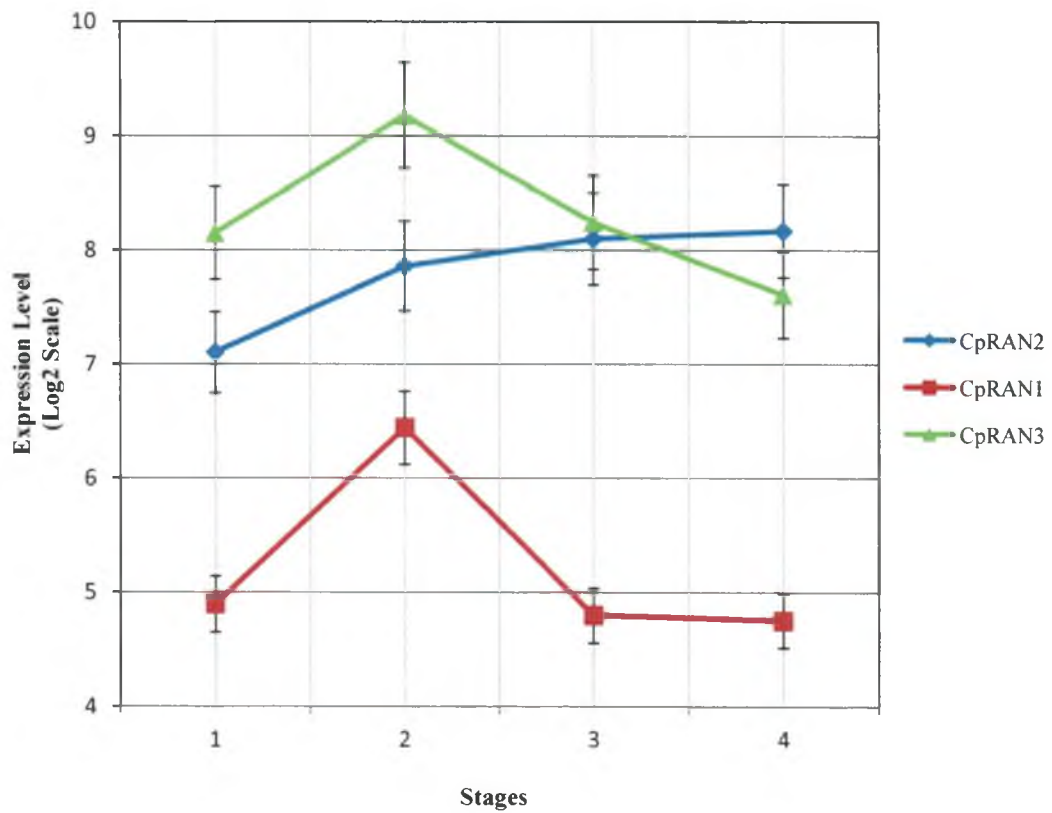
CpRAN, CpCTR, CpEIN2, CpEIN3, CpEIL1, and CpEBF genes at different maturation stages: mature green, 25-30%, 75-80%, and 100% color were clustered. The clustering was done using ArrayStar (DNASTAR, Inc, Madison, Wisconsin, USA) with Hierarchical clustering method based on Euclidean distance metric (linkage method: centroid). This heatmap uses colors to represent the relative value of expressed genes within the given condition. Blue color indicates low expression, yellow indicates intermediate expression and red color represents a relative high expression. The numerical values give the actual values on a log 2 scale and are associated with each color. The color scale is shown as a bar below the figure.



**Figure 20 Heat Map of the expression of ethylene signaling genes in papaya fruit mesocarp during ripening**

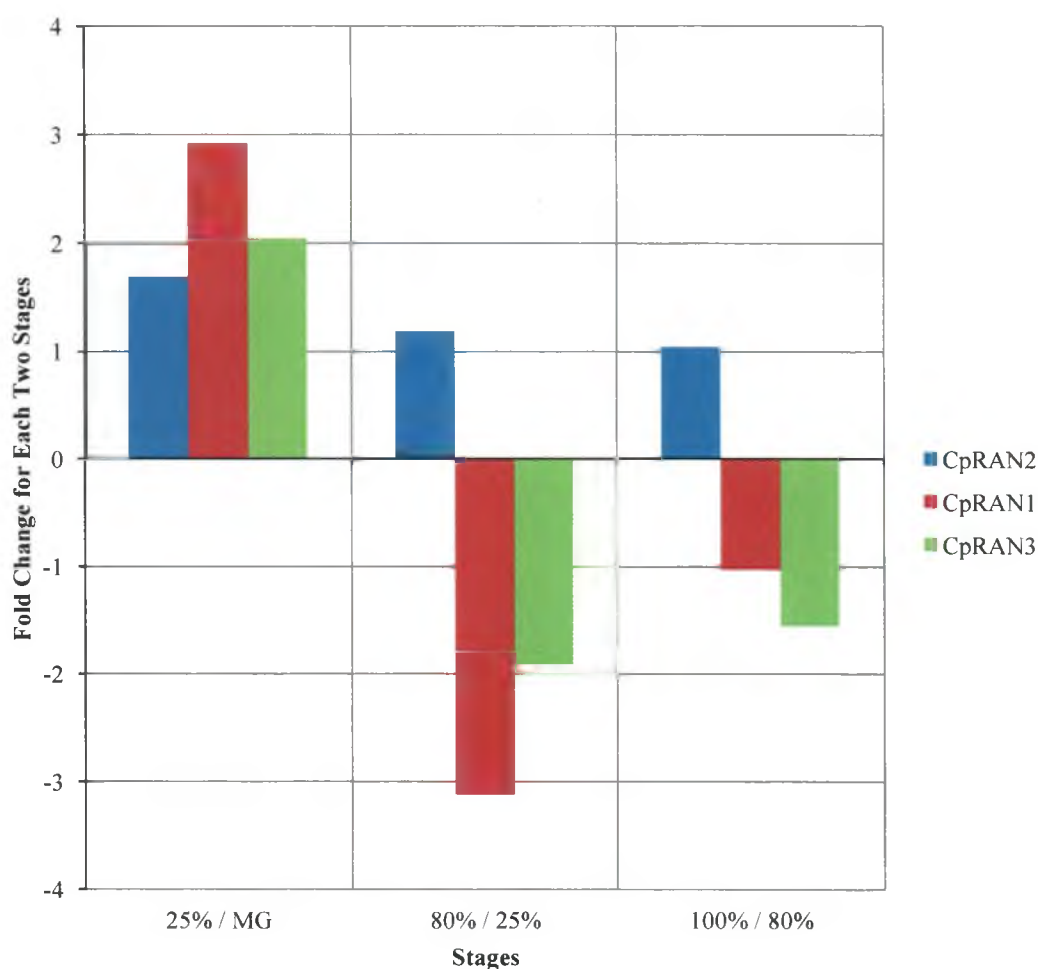
CpRAN, CpCTR, CpEIN2, CpEIN3, CpEIL1, and CpEBF genes at different maturation stages: mature green, 25-30%, 75-80%, and 100% color were clustered. The clustering was done using ArrayStar (DNASTAR, Inc, Madison, Wisconsin, USA) with Hierarchical type and Euclidean distance metric (linkage method: centroid). This heat map used per-row color scaling that used an independent color scale for each row. The values for each gene were displayed as the difference between each column and the experiment baseline (mature green stage). The color scale shown as a bar below the figure and the dot line in histogram above the color scale indicates the zero point for baseline comparison.

## Papaya Signaling Genes -CpRAN-



**Figure 21** Expressions of ethylene signaling gene: CpRAN (Responsive-to-antagonist) in papaya fruit

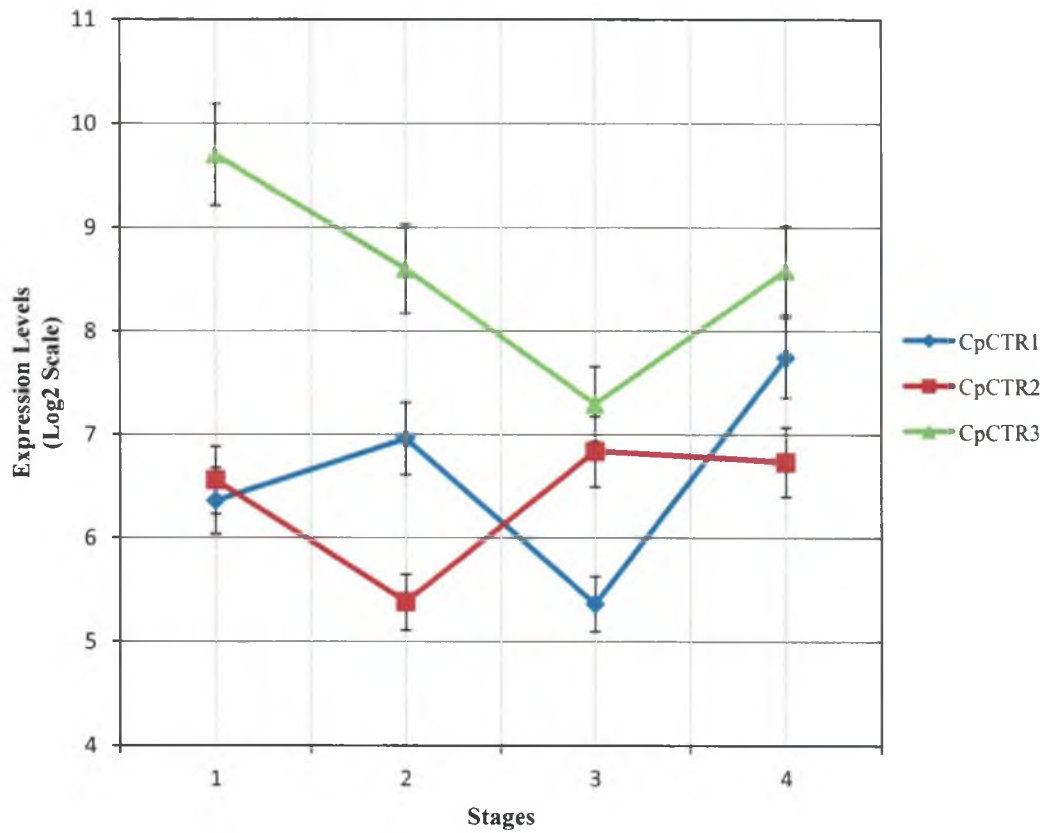
Stages 1, 2, 3, and 4 on x axis indicated mature green (1), 25-30% (2), 75-80% (3) and 100% (4) color stages, respectively. Y axis shows the expression on a log<sub>2</sub> scale (maximum value: 16).



**Figure 22 The fold-change of CpRAN (Responsive-to-antagonist) gene expressions between each two stages**

The “25% / MG” on x axis represented the comparisons of the gene expression at 25% color stage and that in mature green stage (MG). The “80% / 25%” on x axis indicated the comparisons of the gene expression at 80% color stage and that at 25% color stage. The “100% / 80%” on x axis was the comparisons of the gene expression at 100% color stage and that at 80% color stage. The two-stage comparison was estimated gene expression levels on log 2 scale from liner models.

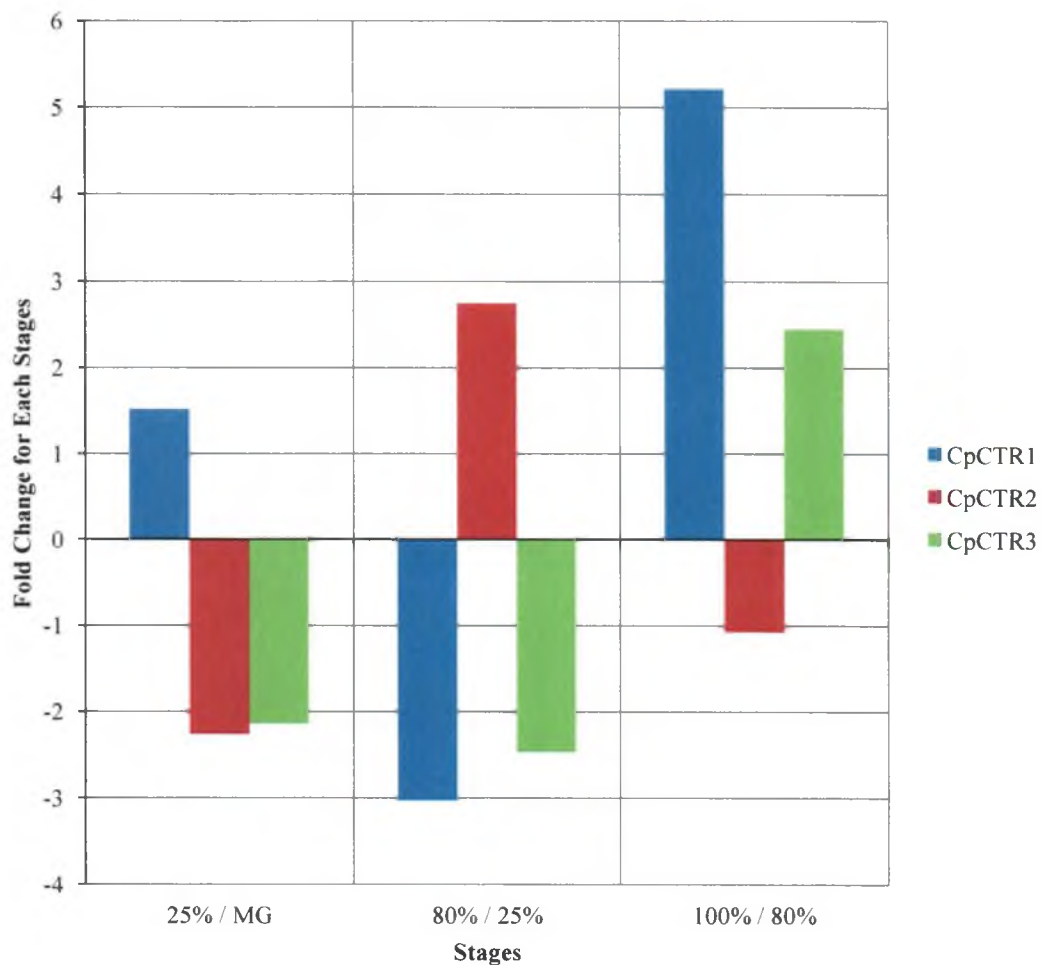
### Papaya Signaling Genes -CpCTR-



**Figure 23** Expressions of ethylene signaling genes : CpCTR (Constitutive Triple Response) in papaya fruit

Stages 1, 2, 3, and 4 on x axis indicated mature green (1), 25-30% (2), 75-80% (3) and 100% (4) color stages, respectively. Y axis shoes the expression on a log 2 scale (maximum value: 16).

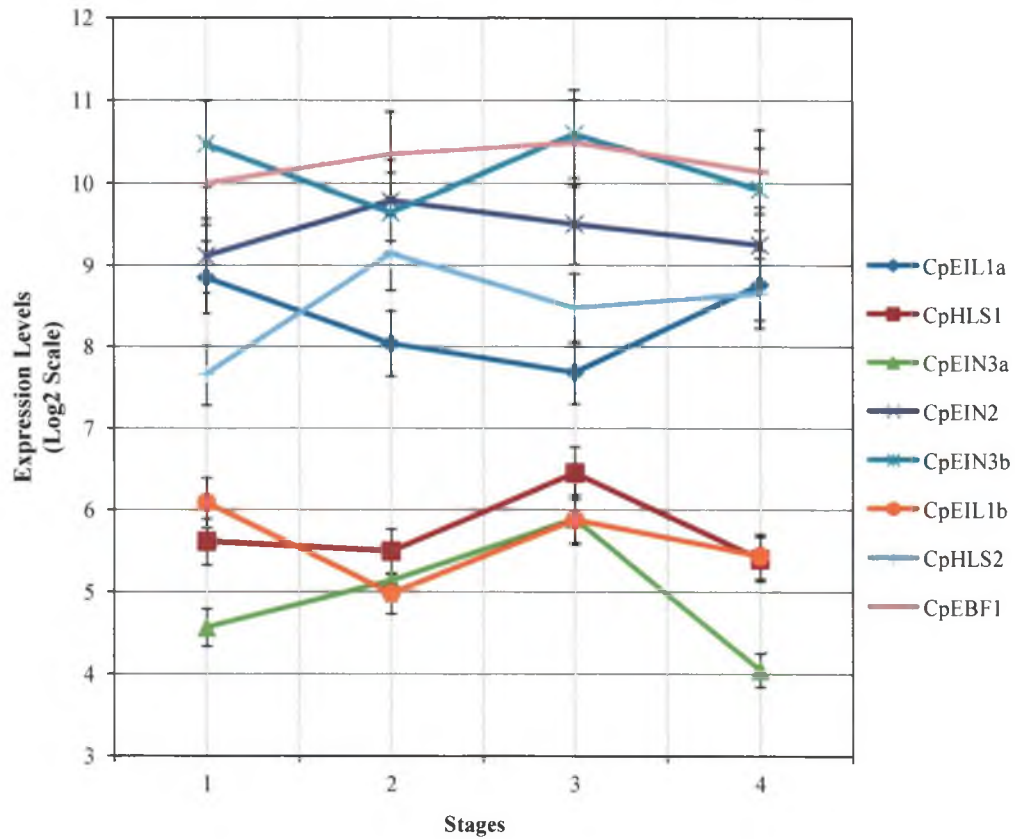




**Figure 24** The fold-change of CpCTR (Constitutive Triple Response) gene expressions between each two stages

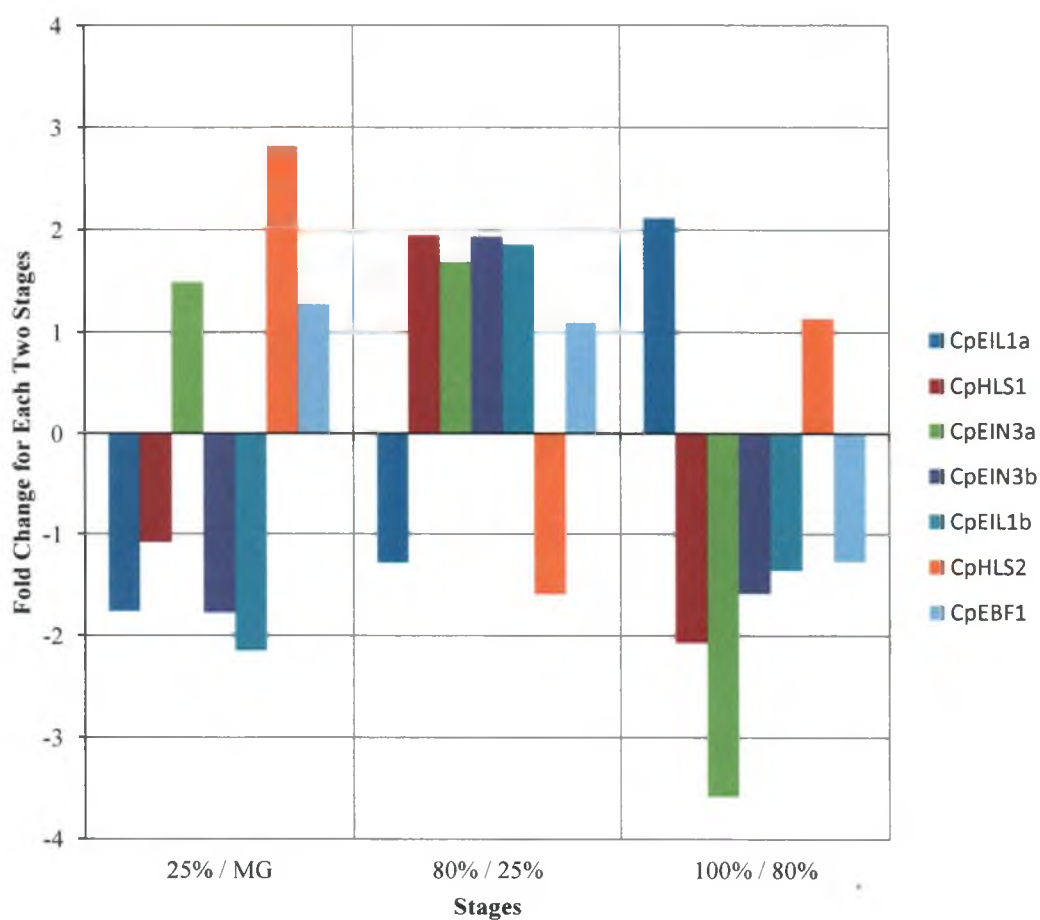
The “25% / MG” on x axis represented the comparisons of the gene expression at 25% color stage and that in mature green stage (MG). The “80% / 25%” on x axis indicated the comparisons of the gene expression at 80% color stage and that at 25% color stage. The “100% / 80%” on x axis was the comparisons of the gene expression at 100% color stage and that at 80% color stage. The two-stage comparison was estimated gene expression levels on log 2 scale from liner models.

### Papaya Signaling Genes -CpEIN2, CpEIN3/CpEIL1s, CpEBF, and CpHLS-



**Figure 25** Expressions of ethylene signaling genes: CpEIN2 (Ethylene Insensitive 2), CpEIN3 (Ethylene Insensitive 3), CpEILs (Ethylene Insensitive 3- like proteins), and CpHLS (Hookless) in papaya fruit

Stages 1, 2, 3, and 4 in x axis indicated mature green (1), 25-30% (2), 75-80% (3) and 100% (4) color stages, respectively. Y axis shows the expression on a log 2 scale (maximum value: 16).



**Figure 26** The fold-change of CpEIN2 (Ethylene Insensitive 2), CpEIN3 (Ethylene Insensitive 3), CpEILs (Ethylene Insensitive 3- like proteins), and CpHLS (Hookless) gene expressions between each two stages

The “25% / MG” on x axis represented the comparisons of the gene expression at 25% color stage and that in mature green stage (MG). The “80% / 25%” on x axis indicated the comparisons of the gene expression at 80% color stage and that at 25% color stage. The “100% / 80%” on x axis was the comparisons of the gene expression at 100% color stage and that at 80% color stage. The two-stage comparison was estimated gene expression levels on log 2 scale from liner models.

## **4. Discussion**

### **4.1 Ethylene Biosynthesis Gene**

#### **4.1.1 Ethylene biosynthesis gene expression during papaya fruit ripening: SAM**

SAM, AdoMet synthase convert the amino acid Methionine to AdoMet (S-adenosyl methionine). In addition to being an essential amino acid for protein synthesis, most methionine is converted to AdoMet, since it is not only a precursor for ethylene biosynthesis, it is also a precursor in the biosynthesis of polyamines, and biotin in plants (Ravanel et al., 1998; Roeder, et al., 2009; Wang, et al, 2002). AdoMet is recognized as an important methyl donor for numerous methylation reactions.

Ripening is usually occurs with a burst of ethylene production in climacteric fruit, and SAM would be expressed before ripening is initiated. Our results showed that the expressions of the three CpSAM were all reduced during the early fruit ripening. The expressions of two CpSAM genes increased when fruit ripening started, and CpSAM3 was the only CpSAM declined during the fruit maturation and ripening. The two SAM isogenes that increased to the 75-80% yellow stage, showed differential expression patterns when they reached the full ripe stage: CpSAM1 increased 4-fold in expression, while the expression of CpSAM4 was less than a 4-fold increased and down-regulated after the 75-80% yellow to 100% fully ripe stage.

It would be reasonable to expect that SAM genes expression would decline at the beginning of ripening and to have increased earlier than the mature green stage to allow the biosynthesis of ACC and ethylene. The biosynthesis of ethylene is auto-inhibited by itself, which means that when the amount of ethylene reaches a threshold, the rate of

biosynthesis of ethylene is reduced (Bapat, et al., 2010; Inaba, 2007). The auto-inhibited reaction may cause the decrease of CpSAM gene expressions.

#### **4.1.2 Ethylene biosynthesis gene expression during papaya fruit ripening: ACS and ACO**

ACC synthesis catalyzes the synthesis of ACC from AdoMet at the start of the ethylene biosynthesis pathway. ACS is considered as a critical enzyme in the ethylene biosynthesis pathway. The last step of ethylene biosynthesis from ACC to ethylene is catalyzed by ACO (ACC oxidase). At least eight ACS isogenes are found in *Arabidopsis* (Argueso, et al., 2007). In tomato, there are nine ACS isogenes and four ACO isogenes have been identified (Barry, et al., 2000; Chang, 2008; Inaba, 2007). Five ACS isogenes have been identified in apple (Tatsuki, 2010). The papaya genome was predicted to have seven ACC synthases (ACS), and three ACC oxidases (ACO) (Paull, et al., 2008).

CpACS1, CpACS2 and CpACS7 were up-regulated at the 25% color stage. Especially CpACS2 showed 25-fold increase from mature green to 25% color stage, while CpACS1 and CpACS7 showed 5-fold and 2.6-fold change, respectively. This increase in gene expressions occurred before the onset of fruit ripening, and may correlate to the burst of ethylene production and which is related to the initiation of fruit ripening. It is noticeable that the expression levels for all three CpACS genes were down-regulated after 25% yellow stage. The peak for ACS gene expression occurred before ripening and then dropped once the fruit ripening process started. Almost all three CpACS gene expressions declined to the base level of expression of the mature green stage except for CpACS2. The expression pattern of CpACS2 showed continuous decline

after the 25% color stage. The decline in gene expression indicated that these three CpACS isogenes may be involved in system 2 ethylene biosynthesis pathway and have a feedback control (auto-inhibition).

The expression patterns of CpACS isogenes showed obvious changes at different maturation stage suggesting that the amount of ACC in papaya fruit accumulated dramatically before initiation and at the onset of fruit ripening and also at the onset of fruit ripening. However, the expression of CpACO isogenes only showed a less than 2-fold change, at a high expression levels throughout fruit maturation. Ethylene's ripening responses is activated once the amount of ethylene reaches a critical threshold. The threshold in papaya fruit could be low, in that way the ethylene responses can be induced when only a small amount of ethylene was reached. The high expression levels of CpACO could lead to the continued ethylene production in papaya fruit at different expression patterns. However, the amount of ACC was controlled by the activities of ACS. The difference in expression between ACS and ACO suggested that they were regulated independently and that ACS was important in ripening initiation.

#### **4.1.3 Ethylene biosynthesis gene expression during papaya fruit ripening: ETO**

Besides the gene expression of ACS and ACO during fruit ripening, recent studies have shown the importance of post-translational regulation of ACS. ETO (Ethylene Overproducer) is known to be a negative post-transcriptional regulator of ethylene biosynthesis in *Arabidopsis* (Wang, et. al., 2002). It has been found that ETO directly interacts with AtACS5 and LeACS3 in *Arabidopsis* and tomato (Yoshida, et al., 2005; Wang, et al., 2002), respectively. In *Arabidopsis*, ETO inhibits the enzyme

activities of ACS (Yoshida, et al., 2005; Wang, et al., 2004), interacts with the conserved C-terminal region found uniquely in type 2 ACS proteins, and degrades ACS via the ubiquitin 26S proteasome pathway (Christians, et al., 2008; Sharkawy, et al., 2008). Both AtACS5 and LeACS3 are recognized as the type 2 ACS isozymes and possess a consensus C-terminal sequence (Yoshida, et al., 2005).

In this research, two homologs of ETO were found in the Papaya Genome. The expressions of both ETO were down-regulated at the 25% yellow stage and up-regulated from 25% yellow to 80% yellow stage. CpETO1 showed a more obvious change from 25% yellow to 80% yellow than CpETO2. Since ETO can inhibit the enzyme activity of ACS, this response could regulate protein degradation via the proteasome pathway, the high expression levels of CpETO could lead to the inhibition of CpACS gene expression at 80% color stage. The three CpACS paralogs (CpACS1, CpACS2, and CpACS7) were up-regulated at 25% yellow stage and then down-regulated at 80% yellow stage with a correlation between the expression levels of CpETO1 and CpETO2. When CpETO was expressed, the gene expressions of CpACS1, 2 and 7 were down-regulated at the same stage. When the expression levels of CpETO were low, the expression levels of CpACS1, 2 and 7 were increased. This suggested that CpACS1, 2 and 7 are type 2 ACS homolog suitable to AtACS5 in *Arabidopsis* and LeACS3 in tomato. It is also parallel they automatically inhibit ACS enzyme activities in papaya fruit.

## **4.2 Ethylene Perception Genes**

### **4.2.1 Ethylene perception gene expressions during papaya fruit ripening**

In papaya, only three ethylene receptors are predicted (CpETR1-3) (Paull, et al., 2008). The fourth receptor, CpETR4, contained an incomplete amino acid sequence and showed low homology with ethylene receptor sequences in other species. However, the expression of CpETR4 showed significant changes between the mature green and 80% yellow stage in papaya fruit in previous experiments (Unpublished). CpETR4 was included in this research and was expected to show the differences during papaya fruit ripening, but only a 2-fold change in CpETR4 gene expression occurred at the 25% yellow stage (Figure 17). The low gene expression and small change during fruit maturation suggested CpETR4 may be a real gene though possibly not an ethylene receptor.

Previous studies have indicated that the amount of ethylene receptor in tomato is correlated to the timing of ripening. An increase in ethylene receptor mRNA occurs with the initiation of tomato fruit ripening (Kevany, et al, 2007). The expressions of CpETR2 and CpETR4 gene increased at the mature green stage to the 25% yellow stage (Figures 16, 17), which agree with the proposal that ethylene receptor may be involved in the control of fruit ripening. The other two papaya ethylene receptors, CpETR1 and CpETR3, showed different gene expression patterns. CpETR1 was down-regulated during early fruit ripening, and then up-regulated from 25% to 80% yellow stage (Figure 18). The gene expression level returned to the base level of expression of mature green stage when the fruit reached the fully ripe stage. CpETR3 had a similar expression pattern to CpETR1. Based on the histidine kinase activity, ethylene receptors can be divided into



two subfamilies: subfamily I and II. In *Arabidopsis*, subfamily I receptors play a more important role in fruit ripening. However, the functions of subfamily I receptors can be substituted by certain subfamily II receptors (Cancel and Larsen, 2002; Hall and Bleecker, 2003; Huang, et al., 2003; Lelièvre, et al., 1997). CpETR2 was the only gene that was up-regulated from mature green to 25% yellow stage. Although it was down-regulated at 80% yellow stage, the overall CpETR2 gene expression was up-regulated throughout fruit ripening from the green mature stage. The gene expressions for other three ethylene receptors were either reduced to the base level (green mature stage) or the change only with a 2-fold increase. These reduces suggested that CpETR2 might play a relative important role in papaya fruit ripening. The other ethylene receptors might be involved in the ethylene responses in different from maturation and ripening stages not covered in this research.

Two papaya ethylene receptors were up-regulated and the other two down-regulated when the fruit was at 25% yellow stage. This suggested that like tomato, the function of ethylene receptors in papaya fruit were complementary to each other independent of the receptor subfamily. As mentioned above, the number of ethylene receptor in papaya is less than in tomato (six) (Lashbrook, et al., 1998; Tieman and Klee, 1999) and *Arabidopsis* (five) (Hua, 1998). The fewer receptors in papaya might suggest that the role of ethylene receptors during papaya fruit ripening may be more important than in tomato. In papaya, two ethylene receptors (CpETR2 and CpETR4) were up-regulated before the fruit ripening was initiated, and could be involving in controlling the timing of fruit ripening. While CpETR1 and 3 were down-regulated (1-2-fold) at the same time, then both were up-regulated at the onset of fruit ripening, suggesting that

these two ethylene receptors may be involved in fruit ripening and possibly inhibit the ethylene response caused by the system 2 ethylene biosynthesis.

### **4.3 Ethylene Signaling and Responses Genes**

#### **4.3.1 Ethylene signaling and response gene expression during papaya fruit ripening:**

##### **RAN1 (Response to Antagonist 1)**

For ethylene binding, the copper ion is an important cofactor. RAN1 (Response to Antagonist 1) plays a central role in transporting copper ions to the ethylene receptors (Rodrigo, et al., 2003). Only one predicted RAN1 gene has been found in the Papaya genome (Paull et al., 2008). In this research, two more genes are likely to be RAN1 paralogs. All three CpRAN1 paralogs were up-regulated at the 25% yellow stage. Two RAN1 paralogs, CpRAN1 and CpRAN3, were down-regulated after 25% yellow stage, and the gene expressions continued to decline until the full ripen stage (Figure 21). Only CpRAN2 was up-regulated throughout fruit maturation and ripening (Figures 20 and 21). Since RAN1 is involved in ethylene binding, the gene expression levels of RAN may correlate to ethylene receptor gene expression during papaya fruit ripening. In Figure 17, two ethylene receptors, CpETR2 and CpETR4, were up-regulated at 25% yellow stage, and then were down-regulated at 80% yellow stage. Low expression levels of CpRAN1 and 3, at the 80% yellow stage may limit in ethylene receptor gene expression. The gene expression level of CpRAN2 increased throughout maturation and ripening process in papaya fruit and was possibly involved in the recovery of ethylene receptor expression after ripening was initiated.

#### **4.3.2 Ethylene signaling and response gene expression during papaya fruit ripening:**

##### **CTR1 (Constitutive Triple Response)**

In *Arabidopsis*, CTR1 (Constitutive Triple Response 1) acts downstream of ETR1 (Ethylene receptor 1), and negatively control ethylene responses. Typically, CTR1 is associated with subfamily I ethylene receptors, for example AtETR1 and LeETR1. Active CTR can inhibit the downstream ethylene responses. In these results, CpCTR2 and CpCTR3 were individually down-regulated initially, and then up-regulated during late ripening (Figure 23). Maintaining the activities of the ethylene receptor and CTR before ripening was initiated can lead to the inhibition of ethylene responses. The gene expression patterns of CpCTR1 and CpCTR2 reach their lowest level was possibly related to the regulation of System 2 ethylene production initiation. All three CpCTR homologs showed the higher expression levels at the 100% color stage which was the fully ripe stage (Figure 11). The relative high expression of CpCTR homolog genes at the 100% color stage may indicate the occurrence of the System 2 ethylene production can activate CTR gene and cause the inhibition in ethylene signaling pathway.

#### **4.3.3 Ethylene signaling and response gene expression during papaya fruit ripening:**

##### **EIN2 (Ethylene Insensitivity 2), EIN3 (Ethylene Insensitivity 3), EILs (EIN3-Like proteins) and EBFs (Ethylene Response Factors)**

The transcriptional factor, EIN2, is thought to be located on the ER membrane and involved in multiple transcriptional regulations for other hormone pathways (Jonson and Ecker, 1998). The *Arabidopsis* ein2 mutant reduces the expression of LeEIN2 and leads to late fruit ripening (Klee, 2004). In the Papaya genome, only one EIN2 ortholog

has been predicted (Paull, et al., 2008), and showed only a slight change at the 25% yellow stage. Since EIN2 could be involved in other hormone response pathways, it was not unexpected to detect no obvious changes of CpEIN2 expression at different ripening stages.

Other transcriptional factors located in the nucleus, CpEIN3 (Ethylene Insensitivity 3) and CpEILs (EIN3-like proteins) are downstream of EIN2 and response for the ethylene responses genes (Jonhson and Ecker, 1998). Over-expression of EIN3 in previous research has induced ethylene response phenotypically, such as the seedling triple response and constitutive ethylene response in adult plant (Chao, et al., 1997; Johnson and Ecker, 1998). Two EIN3 homologs have been predicted in the Papaya genome (Paull, et al., 2008). The differences in gene expression pattern were found between CpEIN3a and CpEIN3b. However, both genes reached the highest expression level at the 80% color stage, and then declined to their base expression level. This pattern suggested that the ethylene responses pathway was most actively at the 80% color stage and were inhibited once ethylene production had been initiated.

Eight ERFs (Ethylene Response Factors) are predicted in the papaya genome. Twelve additional ERF-like genes with low homology matched with ERFs genes in other species were also predicted (Paull, et al., 2008). In this research, only one ERF has been induced in the analysis. The expression level of CpEBF1 was high throughout fruit ripening (Figure 8) after 25% yellow stage by comparison to the gene expression level at mature green stage (Figure 25). This result may indicate that CpEBF1 may be expressed throughout fruit ripening.

#### **4.4 The Significant Differences between Four Stages**

##### **4.4.1 Variations between biological repeats could be caused by biological factors: variations from the plant materials, and the difficulty in recognizing maturation stages of fruit ripening**

Using general linear model analysis the whole data set resulted in the high standard deviation and high raw p-value (data not shown). There were two major reasons possible causes of the high variation between the three biological repeats. First, the sample number was too small to reduce the variation between each fruit. To lower the variation between each fruit, three random selected fruit were combined into one sample, so three biological repeats should contain nine different fruit. However, papaya possibly had high variation and nine samples may not have represented the population. Second, recognizing the specific stages of fruit ripening was another challenge in this research. In papaya, the change of flesh color is much faster than the change in skin color, once the seeds are matured. The only stage with different flesh color is mature green stage. At the mature green stage, the color of skin remained green, seeds were black and flesh color was white to slightly yellow. Estimated the percentage of color on the skin by visually was used in this research. However, slightly differences in the stage could lead to differences in gene expressions and enzyme activities.

##### **4.4.2 Technical error could also cause the high variations**

The quality of sample cDNA was affected by the quality of total RNA, and the mRNA purity. The four stages for each replicate should be randomly applied to different

location on the array. However, four stages for each biological replicate were applied to the same position, which may increase the position effect when scanned.

#### **4.4.3 Multiple probes for one gene**

The NumbleGen offers a 4-plex format with 72,000 probes per array, and our array was designed based on the Papaya genome project which contains 24,421 predicted genes. Results in two to three probes on the array represent one gene. In terms of one probe represents one gene, the intensity of each papaya gene was averaged from two to three data points. Besides this, these two or three probes may react with different part of single gene and may present different intensities for just one gene.

## 5. Conclusions

### 5.1 Genes involved in the inhibition of ethylene biosynthesis and the negative regulations of ethylene signaling transduction pathway were highly expressed, while ethylene biosynthesis and perception genes are relative low in expression at the green mature stage

The papaya green mature stage has full green skin, white to slightly yellow flesh, and black seeds. Fruit at this stage are considered unripe and less sensitivity to ethylene. Papaya fruit at the mature green stage contain high level vitamins and enzymes. After this stage, ethylene sensitivity increases along with ethylene synthesis and the declined of response pathway.

Papaya ETO and CTR homologs showed high gene expression at the green mature stage. ETO in *Arabidopsis* and tomato are involved in the regulation of ACS activity, and could inhibit the biosynthesis of ethylene indirectly. Our results suggested that the expression of papaya ETO homologs maintained high gene expression level before the mature green stage and started to decline after the seeds reached the maturity as judged by the seeds turning from white to black. High ETO gene expression correlated to the low ACS gene expression before and at the green mature stage. Early ACS gene expression began at the mature green stage, and could also be related to the seeds maturation.

CTR is considered to be a negative regulator of ethylene signal transduction pathway in *Arabidopsis*. In papaya, CTR homolog was highly expressed at the mature green stage and any ethylene-dependent responses were potentially initiated until ripening was initiated.

The function of RAN in *Arabidopsis* and tomato is a copper transporter that is associated with subfamily I ethylene receptors. The expression of the papaya RAN homolog increased after the green mature stage, suggested that papaya RAN homologs may be involved in effecting the activities of the receptors CpETR1 and CpETR3. These two papaya ethylene receptor homologs are classified in the subfamily I based upon response alone. These two receptors may play an important role in regulating ethylene signal reception before the onset of papaya fruit ripening.

## **5.2 Ethylene biosynthesis gene expression increased before the initiation of fruit ripening and was followed by an increase in ethylene receptor gene expression**

Papaya fruit at the 25% color stage is still firm. Although only 25-30% of skin had turned yellow, the inside flesh was already fully colored.

The fruit ripening was considered initiated when fruit skin start coloring. CpSAM homologs were involved in the synthesis of ACC precursor, CpETO homolog involved in inhibition of ethylene synthesis, and CpCTR homologs involved in the negative regulation of ethylene signaling pathway declined in gene expression level, while ethylene biosynthesis genes were found to be expressed at the 25% color stage. This suggested the initiation of fruit ripening was not completely regulated by the endogenous ethylene but may be regulated by other factors.

Three CpACS homologs were potentially inhibited by ETO at the green mature stage, dramatical increased in gene expression level at the 25% color stage. The increase in ACS gene expression paralleled the down-regulation of ETO homologs from the green



mature to the 25% color stage. This result suggested that the ethylene precursor, ACC, was produced and accumulated before the 25% color stage. The accumulation of ACC at the 25% color stage may be involved in the System 2 ethylene biosynthesis, a marker for the initiation of ethylene-dependent fruit ripening.

Ethylene receptor gene expression showed similar patterns to that of the ethylene biosynthesis genes. It was difficult to tell the exact timing of ethylene biosynthesis and receptor genes expression. The results indicated that ethylene receptors gene expression was also involved in regulating papaya fruit ripening.

**5.3 The increase in copper transporter (RAN) expression which is associated with ethylene receptors paralleled the increase in ethylene receptor gene expression and occurred before the onset of papaya fruit ripening**

RAN plays an important role in regulation of ethylene receptor activities in Arabidopsis. Three CpRAN were up-regulated at the 25% color stage and suggested the papaya RAN homologs may have been expressed before the initiation of fruit ripening and the occurrence of the System 2 ethylene production, and also play a role in activation of the Subfamily I ethylene receptors

**5.4 The production of System 2 ethylene may activate ETO homologs gene expression, in order to inhibit type 2 ACS expressions. Unlike the early inhibition of ethylene biosynthesis, CTR homolog expressions remain low. The low expression of CTR suggested that ethylene signaling transduction pathway was still active**

The 80% color stage of papaya fruit in this research was determined by the occurrence of ethylene production peak. The ethylene production peak usually appeared after a burst of respiration in papaya fruit. Ethylene biosynthesis might be initiated after the 25% color stage and before the 80% color stage. Only small amount of ethylene were produced at the 25% color stage and that could lead to the System 2 ethylene production occurred in papaya fruit,

Three CpACS homologs expressions declined at the 80% color stage. The declined in papaya ACS homologs gene expressions indicated ACC synthesis was auto-inhibited by large amount of ACC produced at the 25% color stage. The decline of CpETO and CpACS gene expression suggested that ACC synthesis and accumulation may be inhibited by endogenous ethylene at the 80% color stage during papaya fruit ripening.

The other CpACS may be involved in maintaining the base level of ethylene production (System 1). However, the inhibition of ethylene biosynthesis did not directly impair the downstream ethylene signaling transduction pathway. CTR gene expressions in papaya fruit continued to decline at the 80% color stage. Low CpCTR gene expressions suggested that ethylene signaling transduction pathway may still be potentially activated at the 80% color stage.

Gene expressions of CTR homologs continued declined, while the System 2 ethylene biosynthesis began to slow down and maintained the System 1 ethylene

biosynthesis at the 80% color stage which was considered the onset of papaya fruit ripening.

**5.5 The expressions of ethylene related transcriptional factors did not show any significant changes throughout fruit ripening. The changes in gene expression were less than 2-fold and EIN2 protein may not have play a role in the ethylene response**

EIN2 is involved in multiple hormone regulation and its gene expression is maintained at a same level during fruit maturation and ripening. However, in papaya EIN2 gene expression showed a slight increase at the 25% color stage that may indicate multiple responses have been activated or initiated before the onset of fruit ripening.

**5.6 Ethylene responses followed the patterns of ethylene biosynthesis gene expression, and started to decline as the fruit reached the full ripe stage**

EIN3 and EIL are both involved downstream of EIN2 and occur in the nucleus. These two genes are directly involved in controlling ethylene responses. Two EIN3 homologs showed increased gene expression during papaya ripening, and then declined at the 100% color stage.

A decline in the expressions of ethylene signaling pathway genes suggested auto-inhibition of ethylene biosynthesis soon after the onset of System 2 ethylene production. Furthermore, a decline in ACS homologs expressions after System 2 ethylene synthesis initiation also could support the theory of auto-inhibition of ethylene biosynthesis in papaya.

### **5.7 ACC synthase (ACS) seems to play a more critical role in ethylene biosynthesis, further regulating papaya fruit ripening**

ACS and ACO are critical to ethylene biosynthesis. ACS played a more important role in ethylene synthesis and in the regulation on ethylene response during papaya fruit ripening than SAM. The expression of three out of six CpACS homologs increased at or before 25% color stage. ACO gene expressions did not show any dramatic changes during fruit ripening. However, small changes in ACO gene expression did occurred at the 80% color stage, later than the peak in ACS gene expression. This suggested that the accumulation of ACS could be the key regulator of ethylene biosynthesis and the ethylene dependent responses that occurred during fruit ripening. Also, ACC accumulates before the initiation of System 2 ethylene production, and the small amount of change in ACO gene expressions could lead to an increase in ethylene production, further effecting papaya fruit ripening.

### **5.8 Papaya ethylene receptors might initiate ethylene dependent fruit ripening events**

Ethylene receptors are functionally divided into two subfamilies. In *Arabidopsis*, subfamily I ethylene receptors play the more important role in ethylene response. However, subfamily I and II ethylene receptors in tomato seem to substituted for each other. In this research, the function of four ethylene receptors showed no differences in their regulation of papaya fruit ripening. Two different gene expression patterns were found within the four papaya ethylene receptor homologs, and these two patterns were complementary, especially at the 25% color and 80% color stages, which was before the

onset of papaya fruit System 2 ethylene production. The complementary of ethylene receptors functions might lead to their high sensitivity to ethylene, once System 2 ethylene production was initiated.

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